CONTRIBUTIONS TO THE NITROGEN ECONOMY OF AUSTRALIAN WHEAT SOILS, WITH PARTICULAR REFERENCE TO NEW SOUTH WALES.[1]

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(Plate i; five Text-figures.)

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Contents.

Introduction.

Wheat is the most important cereal crop of Australia. The area devoted to its cultivation steadily extended until about 1930, and has for the last decade averaged about 14 million acres, or nearly two-thirds of the total area under cultivation. This wheat area gives an average annual harvest of about 160 million bushels, of which nearly 30% has been produced in New South Wales. The area under wheat for grain in this State has since 1926 averaged about 4 million acres, with an average annual production of about 51 million bushels; the average yield per acre is thus approximately 12·6 bushels. The southern parts of the wheat belt (Riverina and South Western Slopes) in New South Wales contain most of the wheat area.[2]

[1] Approved as a thesis for the degree of *Doctor agronomiae* in the Royal Veterinary and Agricultural College, Copenhagen.

[2] Detailed figures in The Official Year Book of Australia, No. 29—1936, and The Official Year Book of New South Wales, 1935-36. Here also description and map of the wheat belt of New South Wales. Prescott (1934) gives a general survey of the extent and character of the wheat belt in Australia.

The yield of wheat is closely correlated with the rainfall. In South Australia, Prescott (1934) calculated a close and positive correlation between yield per acre and mean rainfall from April to November, and in New South Wales the most fertile wheat districts are found on the Western Slopes in the southern parts of the State, where the average annual rainfall is about 25 inches, with a maximum in winter. Nevertheless the breeding of drought-resistant varieties has made it possible to grow wheat profitably, although with a much lower yield per acre, in districts where the rainfall is as low as 9 or 10 inches.

The common practice in New South Wales is to grow wheat alternating with fallow, or else to interpose a crop of oats for grain, hay or grazing, after the wheat crop. Sowing of the wheat normally takes place in April–May, and harvest in November–December. In harvesting, the heads of the wheat are usually stripped off and the straw burned, either immediately after harvest or else in February–March. The subsequent fallow may be "short", i.e., the ground is ploughed immediately after harvest and stubble-burning, kept bare until May, and then sown with wheat again (common in the northern districts where most of the rain falls during the summer), or "long", i.e., the stubble-ground is not ploughed until the autumn rains begin in May–June, and is then kept bare until sowing-time next year. This is common in the southern districts with winter maximum of rainfall. Spontaneously appearing herbage on the stubble fields is often grazed by sheep, before the fields are ploughed in the autumn. In South Australia and Victoria the wheat or oats crop is often followed by one or two years of pasture, and the stubble-burning may be omitted. This practice is also sometimes followed in New South Wales. Not infrequently the fallowing is omitted and wheat is sown directly on ploughed stubble-land, but as a rule this gives lower yields than sowing after fallow.

It has long been recognized that phosphatic fertilizers regularly improve the yields in the principal wheat-producing areas of Australia, but nitrogenous fertilizers have usually been found to have little or no effect. [Data from earlier experiments in Victoria have been collected by Howell (1911), and more recent data are quoted by Prescott (1934).] In South Australia, Richardson and Fricke (1931) and Richardson and Gurney (1934) found profitable responses from the application of ammonium sulphate or sodium nitrate to cereals sown on stubble land, but not after fallow; they ascribed this difference to the higher nitrate content of the fallowed land. Prescott (1934), in a discussion of the value of fallowing, concludes that the production of nitrate may be more important than the conservation of moisture, to which the good effects of fallowing are often ascribed. In practical wheat farming nitrogenous fertilizers are never used. Since the average nitrogen content of Australian wheat is a little less than 2% and the average weight per bushel some 62–63 lb., the average 12·6-bushel crop in New South Wales thus carries away between 14 and 15 lb. of nitrogen, in the grain alone, from each acre of land. With allowance for the loss of nitrogen caused by stubble-burning, we must reckon with an average consumption of at least 20 lb. of nitrogen per acre per wheat crop, so that the 4 million acres of wheat land in New South Wales annually lose nearly 35,000 tons of nitrogen. To this must be added further quantities represented by the production of oats and of wheaten hay,[3] as well as the nitrogen involved in the production of wool and meat by sheep grazing on the stubble fields.

[3] Wheaten hay is in New South Wales annually grown on an area of about 300,000 acres, with an average production of 1·2 tons of hay per acre.

Unless compensated in some way, this continued removal of nitrogen in the crops must represent a heavy drain upon the nitrogen reserves of the soil, which are not very large in most of the typical wheat areas. Apart from limited areas of heavy black and brown basaltic earths of an almost chernozem-like character in Queensland and northern New South Wales, there are probably few wheat soils that contain more than a few per cent. humus and 0·1 per cent. total nitrogen.[4] Already Howell (1911) has emphatically pointed out the dangers of soil exhaustion by continued wheat cultivation without compensation for the plant food taken away. From the fact that wheat has in some districts been cultivated on the same land for 50–60 years and even more without nitrogenous fertilizers and without any apparent decline in crop yield (as it has been in India for centuries), one might indeed be inclined to conclude that some natural factor compensates the land for the continual removal of nitrogen. But if this is not the case, the nitrogen requirements of the crops must wholly or partially be met by conversion of a part of the humus nitrogen into plant food. This involves a gradual destruction of the humus, eventually leading to unproductiveness and possibly having a close connection with the phenomenon of soil erosion, which in recent years has assumed alarming proportions, not least in the wheat districts. There is therefore an imperative need for a detailed examination of the various factors that influence the nitrogen balance in the wheat soils.

If we leave out of consideration the still unconfirmed statement by Lipman and Taylor (1924) that the wheat plant itself is capable of fixing nitrogen, as well as nitrogen fixation by trees and bushes (Ericaceae, Elaeagnaceae, Casuarineae, etc.), which do not occur on the wheat fields, we have the following natural factors known to be able to add combined nitrogen to the soil:

(1). Rainfall, which brings down small quantities of nitrogen, chiefly nitrogenous oxides produced by electric discharges in the atmosphere;

(2). Nitrogen fixation by leguminous plants, wild or cultivated;

(3). Nitrogen fixation by free-living soil microorganisms.

A fourth factor might be represented by nitrogen fixation through physico-chemical agencies in the soil (Warmbold, 1906; de' Rossi, 1932*c*; Dhar, 1937). The existence of these phenomena, however, still remains to be proved.

The third factor—nitrogen fixation by free-living organisms—has often been considered highly important in soils from warm and arid climates [for references, see Russell (1937); cf. also Kostytchev (1924), Lander and Ali (1925), and Gainey (1932)]. In the interior of New South Wales the rate of evaporation of water practically everywhere exceeds the rainfall except for limited periods in winter time in certain districts; the climate is thus predominantly arid.[5] The process of non-symbiotic biological nitrogen-fixation therefore deserves careful study as a link in the nitrogen problem of the wheat soils of Australia. To undertake such a study on systematic lines has been the scope of the present work. When this was started towards the end of 1935, there were in the literature only few contributions dealing with the occurrence of nitrogen-fixing bacteria in Australian soil and practically no experimental work had been done on the quantitative aspect of this problem.

[4] Detailed descriptions of the soil types of Australia in general, and New South Wales in particular, are due to Prescott (1931) and Jensen (1914).

[5] Official Year Book of New South Wales, 1935-36. Cf. also Richardson (1923) and Richardson and Fricke (1931) on rainfall and rate of evaporation in Victoria and South Australia.

The Microorganisms Active in Non-symbiotic Nitrogen Fixation.

The investigations of Berthelot (1888–90) from 1885 onwards were the first to deal rationally with the problem of fixation of elementary nitrogen by micro-organisms not living in symbiosis with a host plant. Although it may nowadays, as pointed out by Waksman (1932), appear open to doubt whether nitrogen fixation actually took place in Berthelot's experiments, these investigations proved highly fruitful by stimulating interest in the problem, and opened up an ever-increasing flow of contributions to the problem of microbial nitrogen-fixation.

The microorganisms capable (or allegedly so) of fixing elementary nitrogen without symbiosis with a host plant are:

1. *Aerobic organisms.*—The most important group of these is the bacterial genus *Azotobacter*, first discovered by Beijerinck (1901). The genus includes four well-defined species, *Az. chroococcum*, *beijerinckii*, *vinelandii*, and *agile*, of which the first is by far the most common in the soil. A fifth species, *Az. indicum*, has recently been added by Starkey and De (1939).

The group has been studied very thoroughly from different angles,[6] and in recent years Burk and co-workers (1930–36) have given numerous important contributions to its physiology. The main points may be summarized thus: The *Azotobacter* are strictly aerobic organisms, although still capable of growth at quite low oxygen tensions (Meyerhof and Burk, 1928). Their temperature range extends from about 10°C. to about 40°C., with an optimal zone at 30–35°C. They are strongly sensitive to acidity, with optimum at pH 7·0–8·0. A wide range of organic compounds may serve as carbonaceous food—fatty and oxy-acids, higher and lower alcohols, mono-, di-, and polysaccharides, etc. (Mockeridge, 1915; Gainey, 1928; Winogradsky, 1932). The actual fixation of nitrogen is brought about by a highly specialized system of enzymes, *azotase* (Burk, 1934), of which a specific component, *nitrogenase*, is capable of combining directly with elementary nitrogen. This enzyme-complex has so far, except for a still unconfirmed statement by Bach et al. (1934), resisted all attempts at isolation, and its production and activity appear inseparably linked with the synthesis of cell substance. Burk (1934) therefore describes it as "growthbound", and suggests the name *phyo-enzymes* for this type of enzymes. The nitrogenase requires a certain concentration of calcium (or strontium) for its functioning, is incapable of any activity at reactions below pH 6·0, and is strongly activated by even minute concentrations of molybdenum or to a lesser degree vanadium. The primary product of nitrogen fixation is not precisely known; that it should be ammonia, as supposed by Winogradsky (1932), is not definitely proved (Burk and Horner, 1936). According to Endres (1935) and Virtanen and Laine (1937), an oxime compound seems involved. Most of the fixed nitrogen is present in the cultures as cell material, and only small quantities of combined nitrogen (Endres, 1935; Roberg, 1935; Virtanen and Laine, 1937), or none at all (Meyerhof and Burk, 1928), are secreted into the medium as long as growth takes place. When the medium is exhausted of nutrients and growth has ceased, a rapid production of ammonia from the cell material sets in (Roberg, 1935; Burk and Horner, 1936).

Besides elementary nitrogen, *Azotobacter* can assimilate nitrate, ammonia, and simple amino compounds. All these compounds interfere in a remarkable way

[6] No attempt is made here to review the whole literature on *Azotobacter* and nitrogen-fixing bacteria in general. Reference may be made to de' Rossi (1932*c*), Waksman (1932), Burk (1934), and Russell (1937).

with the fixation of free nitrogen, apparently not merely by preferential assimilation, but by actual inactivation of the nitrogenase; Burk and Lineweaver (1930) observed this effect already at concentrations of 0·5 mgm. NO_3– or NH_4–N per 100 c.c. of medium. Bortels (1936) claims that with certain concentrations of molybdenum or vanadium (the action of which latter appeared to be supported by wolfram) nitrogen may still be fixed in the presence of higher concentrations of combined nitrogen. If this is generally valid, it will have an important bearing on our conceptions of the activity of *Azotobacter* under natural conditions.

Azotobacter transforms carbon compounds practically quantitatively into carbon dioxide, water, and cell substance. The amount of nitrogen fixed is generally close to 10 mgm. per gm. of sugar consumed, and other carbon compounds are utilized with an efficiency roughly proportional to their heats of combustion (Gainey, 1928). The efficiency of nitrogen fixation, however, varies with the type of organism as well as with experimental conditions, as we shall discuss in detail later.

Stapp and Bortels (1936) claim to have observed a remarkable influence of weather conditions on the growth of *Azotobacter* in pure culture; it remains uncertain whether this "weather-factor" also operates under natural soil conditions.

Numerous spore-forming and non-spore-forming bacteria, as well as actinomycetes, yeasts and filamentous fungi, have been claimed to fix small quantities of nitrogen, not always on good evidence. In some cases (Bondorff, 1918; Selim, 1931) the gains of nitrogen indeed seem too large to be explicable as analytical errors, but many other claims seem less convincing. For instance, Löhnis and Pillai (1905–08) reported nitrogen fixation in many bacteria, among which they include *Bact. radicicola*, which, according to more recent studies (M. Löhnis, 1930), has never been proved to fix nitrogen outside the host plant. The source of error which M. Löhnis pointed out (loss of nitrogen from the sterile control medium) might have been present in many other cases, and many of the claims of nitrogen fixation by aerobic bacteria other than *Azotobacter* may therefore be regarded with scepticism (cf. de' Rossi, 1932*c*). That actinomycetes and fungi (apart from the species of *Phoma* producing mycorrhiza in the Ericaceae) are unable to fix nitrogen in ordinary laboratory media can be regarded as fairly certain (Roberg, 1931; Waksman, 1932). Some organisms of these groups, as well as numerous bacteria, have been claimed by Emerson (1917), Carter and Greaves (1928), J. D. Greaves (1929–31) and Greaves and Greaves (1932) to fix nitrogen in sterile soil, although not in solution media. Greaves and Greaves (1932) make the significant remark that the aggregate effect of large numbers of such weakly nitrogen-fixing organisms may be much more important than that of a few highly effective forms like *Azotobacter*. These experiments, however, have one patent weakness in common: no allowance was made for the possible loss of nitrogen from the sterile control soil during incubation (Pfeiffer et al., 1906; Warmbold, 1908), which loss might not have taken place in the inoculated soil (cf. M. Löhnis, 1930). Still the possibility of such a non-specific nitrogen fixation must be regarded as existing, and deserves attention.

No attempts have been made to explain the mechanism of nitrogen fixation in these organisms; Winogradsky (1926–28) suggests the possibility of a rudimentary power of fixation due to the general catalytic properties of microbial protoplasm—which may indeed be quite significant if common to a large proportion of the total soil microflora.

Algae as primary agents of nitrogen fixation have been the subject of much controversy. Previous statements on nitrogen fixation by green algae have been fully disproved by Bristol and Page (1923), who still admit that their work does not actually prove the non-existence of nitrogen-fixing algae. More recently, Drewes (1928), Allison and Hoover (1935), Winter (1935), and De (1939), have shown beyond any doubt that certain blue-green algae are capable of fixing nitrogen. The mechanism of fixation is totally unknown.

2. *Anaerobic organisms.*—Nitrogen fixation was first definitely proved in *Clostridium pasteurianum* by Winogradsky (1895), and was later shown by Bredemann (1909*b*) to be a common property of the butyric acid bacilli. Some of these organisms are strict anaerobes, while others are less sensitive to oxygen (Pringsheim, 1906). Although more tolerant of acidity than *Azotobacter* (Dorner, 1924; Willis, 1934), they yet have a definite optimum at approximately neutral reaction (Dorner, 1924). In pure cultures generally 2 to 3 mgm. N are fixed per gm. of sugar fermented, although some strains may fix 5 to 6 mgm. (McCoy et al., 1928), or even more (Kostytchev, 1924). The mechanism of fixation is not well known, but is often explained as a direct reduction of elementary nitrogen to ammonia by nascent hydrogen.

A facultative anaerobe, *Bac. asterosporus*, was shown by Bredemann (1909*a*) to fix small amounts of nitrogen—1 to 3 mgm. per gm. of sugar fermented. It seems uncertain whether this faculty is also displayed under strictly aerobic conditions.

The observations of Clausen (1931) on an exceptionally vigorous nitrogen fixation by anaerobic cellulose-decomposing bacteria still remain to be confirmed.

Of all these organisms, *Azotobacter* is generally assumed to be the most important under field conditions, although some authors, e.g. Bonazzi (1924), have suggested that the clostridia may really be equally or more important by virtue of their wider distribution in soils (Bredemann, 1909*b*) and their greater resistance to acidity.

Earlier Investigations on Non-symbiotic Nitrogen Fixation in Field and Pot Experiments.

Soon after the fundamental experiments by Berthelot and the first isolation of the bacteria in question by Winogradsky and Beijerinck, attempts were made to assess the importance of this process in the nitrogen economy of the soil, partly on more theoretical grounds, and partly on the basis of observations from vegetation experiments.

Among the theoretical estimates, we may mention that of Remy (1909), who calculated that a soil containing 2% humus might annually fix 50 kgm. N per hectare, under the assumption that one-eighth of all the soil humus were annually transformed by *Azotobacter*. Löhnis (1909) estimated that a fixation of some 40 kgm. N per hectare per annum might be expected, *provided* the soil received an annual supply of 4000 kgm. organic matter per hectare, *and* that all this were utilized by bacteria capable of fixing 1 part of nitrogen per 100 parts of organic matter consumed. (As mentioned above, Löhnis reckoned with a large variety of soil microorganisms as nitrogen-fixers.) He pointed out, however, that this represents an upper limit, and that the actual gain might well drop to 10 kgm. or less. Alway and Pinckney (1909), in a similar way, arrived at a more conservative estimate of 8 lb. N per acre annually. Among recent authors, Winogradsky (1932) confines himself to the qualitative statement that "le rôle fertilisant des

Azotobacters, producteurs d'ammoniaque synthétique, ne saurait être mise en doute" (l.c., p. 299), whereas Bonazzi (1924) regarded the process as displayed only under conditions of nitrogen starvation, and de' Rossi (1932*b*–*c*) in Italy, Beck (1935) in South Australia, and Lochhead and Thexton (1936) in Canada, also take a sceptical view, all pointing out the low numbers by which *Azotobacter* is normally represented in the soil. Clearly all such speculations will carry little weight unless supported by direct evidence of the actual gain of nitrogen.

Many attempts have been made to estimate such gains under field conditions, either by simple observations on crop yields, by periodical nitrogen determinations in one and the same soil, or by comparing the nitrogen content of cultivated and uncultivated soils of similar character.

Kühn (1901), in Germany, believed that he found evidence of vigorous nitrogen fixation in soil under permanent rye cultivation, since the crop yields did not decrease during 30 years of cultivation without nitrogenous fertilizers. Pfeiffer (1904) criticized these conclusions severely and pointed out the difficulty of distinguishing between nitrogen recuperation and simple depletion of the soil's own store of nitrogen. The much older experiments on Broadbalk, Rothamsted, show, in a plot under permanent wheat cultivation without nitrogenous fertilizers, a close balance between (*a*) nitrogen removed in the crops and (*b*) nitrogen added by rain and seed, plus decrease in nitrogen content of the soil, which seems to have come to an equilibrium at about 0·1% N (Hall, 1905*b*; Russell, 1937). According to Hall (1905*b*), the plot also loses (or did at that time lose) about 10 lb. N per acre annually with the drainage water, which loss thus seems to be covered by nitrogen fixation. This fixation may in part be due to leguminous weeds, especially *Medicago lupulina*. Hall therefore considered it doubtful whether non-symbiotic nitrogen fixation assumed significant proportions in arable soils. But in soil that had been left undisturbed for 19 years and carried a heavy vegetation of grass and very few legumes, Hall (1905*a*) observed a gain of nitrogen corresponding to at least 25 lb. per acre per annum.

Besides these fundamental observations and theoretical estimates (whence probably the frequently encountered statement in text-books that "non-symbiotic nitrogen fixation may be expected to add from 15 to 40 lb. N per acre per annum to the soil"), most data have come from the United States and from India, in which latter country wheat cultivation without nitrogenous fertilizers is ancient practice.[7]

Alway (1909) found large decreases in nitrogen content of Nebraska soils under wheat cultivation for 9 to 30 years, in comparison with virgin soils. The same was observed in soils from Saskatchewan, Canada, by Alway and Trumbull (1910). Upon the whole it is common experience that cultivation of the rich prairie soils of Canada is accompanied by great losses of nitrogen (Russell, 1937).

Bradley and Fuller (1910) found no appreciable difference in nitrogen content of Oregon soils under wheat cultivation for 17 to 30 years, and adjacent uncultivated soils. They assumed that nitrogen fixation maintained a nitrogen balance in the soil.

Stewart (1910) and Greaves (1914), in Utah, arrived at similar results, and also offered the activity of *Azotobacter* as an explanation. In another paper Stewart and Hirst (1914) suggest the possibility of utilization of nutrients in

[7] All the data discussed here refer to soils not fertilized with nitrogen, unless otherwise stated.

the subsoil, due to deep root development under arid soil conditions, and deposition of organic matter from plant residues in the surface soil.

Swanson and Latshaw (1919), in Kansas, found that soils cultivated for up to 45 years had lost nitrogen in comparison with uncultivated soils, but the losses were smaller under arid than under humid conditions. (No figures were given for the crops.)

Gainey et al. (1929) analysed a large number of soils in Kansas with 12 years' interval and found a general tendency to loss of nitrogen, which loss appeared small or insignificant in soils with a low nitrogen content (0·08–0·12% N). This was especially noticeable under wheat or barley, continuous or alternating with fallow. The authors conclude that a balance of nitrogen tends to become established at about 0·1% N (cf. Hall, 1905*b*, and Russell, 1937), where the losses of nitrogen were supposed to be compensated by the activity of *Azotobacter*.

Jones and Yates (1924), in Oregon, and Metzger (1936), in Kansas, observed strong decreases in nitrogen content of soils under permanent cereal cultivation. Metzger calculated an average annual decrease in soil nitrogen of 0·64% under permanent wheat and 2·28% under permanent maize. The loss was greatest in the first years of the experiment, and an equilibrium also tended to establish itself here.

Holtz and Vandecaveye (1938), in Washington, in a permanent fertilizing experiment found large losses of nitrogen from N-fertilized plots after 13 years, but considerable gains (allowing for the nitrogen carried away in the crops) in a few cases (control plot and plot receiving 2700 lb. straw per annum under permanent wheat cultivation), which apparent gains they ascribe to the activity of *Azotobacter*. Under alternating wheat and fallow there was a general tendency to loss of nitrogen besides that removed in the crops.

All these observations apply to the more or less arid parts of North America. In the more humid regions (New York State), Lyon and Wilson (1928) and Lyon and Bizzell (1933–34) observed gains corresponding to 17 to 38 lb. N per acre annually in permanent plot experiments of 9 to 10 years' duration, either under permanent grass or in legume-free-rotations. The gain was highest where the grass was mown annually and allowed to remain on the ground (cf. Hall, 1905*a*).

Salter and Green (1933), in Ohio, calculated that soil under permanent oats or wheat for 30 years lost annually 1·5% of its total nitrogen, and under permanent maize even twice this amount.

Prince et al. (1938), in New Jersey, observed an increase in N-content from 0·097 to 0·116% in plots fertilized with P and K and abandoned to weed growth for 24 years. Part of this gain, which corresponds to a total of 380 lb. N per acre in 2 million lb. of surface soil, or an average of 16 lb. per acre per annum, seemed to be due to wild leguminous plants.

In India, Wilsdon and Ali (1922), Lander and Ali (1925), Annett et al. (1928), and Sahasrabuddhe et al. (1932–36) carried out shorter or longer series of periodical nitrogen determinations in soil. Marked fluctuations in the nitrogen content were observed by all these authors, and were interpreted as alternating gains (by non-symbiotic fixation) and losses (by denitrification or by leaching). Sreenivasan and Subramanyan (1934) found similar fluctuations, but did not ascribe them to biological causes.

In Nigeria, Diamond (1937) observed the same phenomenon within few days and even hours. He pointed out that no soil microorganisms yet known could have brought about such changes.

This view is not shared by Fehér et al. (1936–37)[8] in Hungary; the fluctuations in nitrogen content of soil reported here are so extraordinary (sometimes several hundred per cent. within a few months!) that it seems extremely hazardous to correlate them with the changes in the comparatively low numbers of soil micro-organisms in general and nitrogen-fixing bacteria (the nature of which was not closely specified) in particular. It seems obvious that if non-symbiotic nitrogen-fixing bacteria could accomplish such effects, all use of nitrogenous fertilizers would be purposeless.

Bortels (1937), in Germany, mentioned an increase in humus and nitrogen content of plots treated for 4 years with molybdenum and vanadium salts, and ascribed the effect to the stimulation of *Azotobacter* by these salts. No detailed figures were given.

Finally we may mention an observation by Kostytchev (1924) on a Crimean soil that had produced good crops of tobacco for 35 years without nitrogenous fertilizers, and similar observations by Loew (1927) on soils from Brazilian coffee plantations. Both authors stress the importance of *Azotobacter* under tropical soil conditions, but give no quantitative data.

In Australia, the only work of this kind so far published is due to Howell (1911), who compared the nitrogen content of 9 Victorian wheat soils, cultivated for 6 to 30 years, with that of corresponding virgin soils, and found in most cases evidence of a loss of nitrogen far in excess of the amount carried away with the crops. In soils with a low nitrogen content (about 0·1% N) there was some indication of a nitrogen equilibrium having become established.

The evidence from the field experiments and observations is thus conflicting and difficult to evaluate, owing to the many sources of error involved. Probably the most important among these is the sometimes surprisingly large variability in the composition of the soil, as shown by Pfeiffer and Blanck (1912) in Germany, Prince (1923) in New Jersey, and Sreenivasan and Subramanyan (1934) in India, besides others (cf. also Thiele, 1905). Further disturbing factors are the losses caused by leaching of nitrates from the soil (varying, of course, with the precipitation) and by wind or water erosion, as well as gains represented by nitrogen brought down by the rain or fixed by wild leguminous plants. Finally there is the analytical error in the actual nitrogen determination, which can sometimes be considerable (Pfeiffer et al., 1906). All these circumstances in connection with the fact that exhaustion of the nitrogen reserves of a normal soil is a very slow process (Russell and Richards, 1920) make it difficult to decide whether the tendency to establishment of a constant nitrogen level, so often observed, represents a real equilibrium between gains and losses of nitrogen, or whether it means that the rate of loss is getting continually slower. The only fact that seems to be definitely established is, that rich soils lose nitrogen at a faster rate than poorer ones. And even if a permanent nitrogen-equilibrium has been established in the soil, most of the field observations give us no information on the relative amounts of nitrogen gained from the rain, from non-symbiotic nitrogen fixation, and from wild-growing legumes. Observations by Howard (1906) suggest that the last factor may in India be of much more importance than is usually appreciated, and the same may be the case in other countries, including Australia (cf. Howell, 1911).

[8] "Die Regenerierung des Gesamtstickstoffgehalts des Bodens wird nach unserer Ansicht nur durch die Tätigkeit der stickstoffbindenden Bakterien ermöglicht" (Fehér and Frank, 1936).

In pot experiments, which may be taken in a wider sense to include experiments in cylinders or masonry frameworks, most of these sources of error are ruled out, particularly the soil variability, and many important studies on the nitrogen economy of the soil have been carried out in this way. The most important papers referring to non-symbiotic nitrogen fixation are the following:

Richter (1899) grew successive crops of oats and mustard, and found evidence of a certain gain of nitrogen, which became noticeable as the content of available soil nitrogen decreased.

Voorhees and Lipman (1905) found small gains of nitrogen (about 3% of the initial) in unmanured soil planted with oats, and larger but irregular gains, even up to 34%, in soil with farmyard manure and planted with millet.

In cylinder experiments on open field, Lipman and Brown (1908) found no positive evidence of nitrogen fixation. Lipman and Blair (1920), in similar experiments continued for 20 years, again found no indisputable gains of nitrogen in cylinders where no legumes were grown, although there was some evidence that the nitrogen content approached a constant level in unfertilized cylinders where the crop yields were very low.

Pfeiffer et al. (1909–10) carried out extensive pot experiments with sterilized or untreated soil, unplanted or planted with oats or mustard. In the first series (1909) no indisputable gains of nitrogen were found, but in the second series (1910) the gains were considerable, averaging 1·034 gm. N per 14 kgm. of soil, or about 5% of the total N-content. The authors emphasize that such large gains could not be expected under field conditions. (It seems highly desirable that such experiments should be repeated on soils of different character and under different climatic conditions.)

In a later series of experiments conducted for 12 years with soil in masonry frames, Pfeiffer (1921) observed no gains of nitrogen by non-symbiotic fixation.

Gerlach (1934) carried out similar experiments for 12 years, using an artificial soil consisting of sand, kaolin, and mineral nutrients. The crop yields gave no indication of nitrogen fixation in untreated soil not carrying legumes, whereas some nitrogen was gained in soil receiving periodical dressings of glucose. Unfortunately no nitrogen determinations were made on the soil medium itself.

Lemmermann et al. (1910) carried out pot experiments with mustard and found small but significant gains of nitrogen, which amounted to 1 to 4% of the total nitrogen content of the soil, but which were smaller than the quantities of nitrogen taken up by the plants.

Wright (1920) conducted pot experiments with different soils and crops for 1 to 3 years, and found evidence of loss rather than gain of nitrogen under fallow as well as under non-leguminous crops.

A kind of transition between field and pot experiments is represented by the lysimeter experiment at Rothamsted (Russell and Richards, 1920): a soil kept free from vegetation for 47 years showed no evidence of gains of nitrogen other than that brought down by the rain, or losses other than through leaching of nitrate.

A special category of experiments is represented by those cases where nitrogen fixation has been artificially stimulated by addition of sugar to the soil. That notable gains of nitrogen and corresponding increases in crop yield can be obtained in this way was first shown in pot experiments by Koch et al. (1907) and Remy (1909), and later by Hutchinson (1918) who also showed that similar results could be obtained under field conditions (cf. also Russell, 1937); such use of

molasses as an indirect nitrogenous fertilizer has in recent years attracted considerable attention (Dhar et al., 1936–37; Russell, 1937). Even in pot experiments, however, this effect of sugar is not constant (Pfeiffer et al., 1910), and the same applies to experiments where less readily assimilable organic materials have been added in order to stimulate fixation. Hutchinson (1918) thus found large gains of nitrogen in sand culture experiments with addition of hay dust and inoculation with a mixture of *Azotobacter* and putrefactive bacteria. Heinze (1926) reported increased yields of oats in pot experiments with additions of straw to the soil and inoculation with *Azotobacter* and various microbial preparations. A complete nitrogen-balance was not reported. Koch and Rippel (cit. after Waksman, 1932) found no gain in nitrogen in 10-year pot experiments with addition of filter paper or straw to the soil, although sugar gave positive results.

In connection herewith, we might mention some sand culture experiments by von Caron (1934), indicating gains of nitrogen by barley inoculated with certain bacteria not closely specified, and experiments by Truffaut and Bezssonoff (1925), who claimed to have grown maize to maturity in sterile sand medium inoculated with a mixture of nitrogen-fixing bacteria. None of these experiments appear well documented, and the results do not so far seem to have been corroborated by other investigators.[9]

The evidence from these experiments is thus conflicting, like that from the field observations. Although it is certain that nitrogen fixation can be induced by adding sugar, etc., to the growth medium, we are still left in doubt as to the amount of fixation that may take place under natural conditions where such high concentrations of easily assimilable nutrients are not present. Also, the construction of a complete and reliable balance-sheet of nitrogen is a difficult matter, and it is not always clear whether the difficulties involved in making reliable determinations of the nitrogen content of large quantities of soil have been overcome (cf. Pfeiffer et al., 1906–09).

In both field and pot experiments the work has been predominantly chemical, and only in few cases (Dhar and Seshacharyulu, 1936; Fehér et al., 1936–37) have observations on the abundance of nitrogen-fixing bacteria been combined with the chemical analyses.

We now arrive at the pure laboratory investigations on nitrogen fixation, which exist in an almost overwhelming number, and which, besides pure culture studies on the morphology, physiology and taxonomy of nitrogen-fixing microorganisms, chiefly deal with (1) the distribution and numbers of nitrogen-fixing bacteria in soils under natural conditions, and (2) estimation of the "nitrogen-fixing power" of soils under laboratory conditions. Many investigations in this second direction have in the past been carried out by means of the solution method first introduced by Remy; the principle of this method is to inoculate a selective liquid medium with a definite quantity of the soil to be examined, and to determine the amount of nitrogen fixed after an arbitrary period of incubation. Although the gain of nitrogen can be expressed in terms of organic nutrients (usually sugar or mannite) consumed, the method gives little information on what would happen in the soil itself. When all necessary mineral nutrients are supplied, the method shows little beyond the mere presence or absence of nitrogen-fixing bacteria in the soil; if nutrients are omitted and nitrogen-fixing bacteria

[9] As to the general aspects of the now chiefly historically interesting problem of inoculation with other than root-nodule bacteria, see Waksman (1932). Some recent work in this direction by Russian investigators is abstracted by Starkey (1938).

artificially added (the inoculation principle of Christensen, 1915), the method may give information on the soil's supply of available mineral nutrients, especially phosphate, and has come into some use for this purpose. As a method for studying the nitrogen-fixing capacity, it is, not least due to the trenchant criticism of Winogradsky (1925–26), more and more becoming replaced by the soil method, in which a certain quantity of soil, with or without addition of organic materials, nutrient salts, etc., is incubated for a certain length of time and the resulting change in nitrogen content determined by chemical analysis of the soil before and after incubation. This was the principle in the pioneer work by Berthelot (1888–90) and Schloesing (1888), and it was nearly 20 years before the method was again applied systematically by Schneider (1906), Warmbold (1906), Koch et al. (1907) and Remy (1909). This method has the advantage of coming closer to natural soil conditions than the solution method, and its most severe limitation is the difficulty of detecting changes smaller than one or two per cent. of the total nitrogen content of the soil.

A number of important contributions have been published by Winogradsky (1925–32), whose methods of "spontaneous cultures", however, aim chiefly at determining the nature and density of nitrogen-fixing bacteria present rather than the amount of fixation which they bring about in the soil itself. The method of determining the relative nitrogen-fixing power of soils on big plates of silicic acid gel with mannite (Winogradsky, 1926–28) really only differs from the solution method in being more strictly selective for *Azotobacter* and in giving quantitative expressions for the relative density of this organism.

Experimental.

All nitrogen-fixing microorganisms, with exception of the blue-green algae, require organic compounds as sources of energy; in Australian wheat soils, the chief groups of organic materials that might come into consideration, are the following:

i. The structureless organic matter of the soil ("humus"). According to the present state of our knowledge, this is largely unavailable to the nitrogen-fixing organisms.

ii. Straw, stubble and roots of cultivated plants (especially wheat and oats) and weeds.

iii. Organic compounds secreted by the roots of higher plants.

iv. Organic compounds elaborated by lower photosynthetic plants (algae).

If the nitrogen requirements of the wheat crops must be compensated by non-symbiotic nitrogen-fixation, we should expect nitrogen-fixing organisms to figure prominently in the transformation of the rather limited quantities of these materials and actually to derive their nitrogen from the atmosphere—or else autotrophic nitrogen-fixers (blue-green algae) must frequently find favourable conditions for active growth and nitrogen-fixation.

A fixation of merely 20 lb. nitrogen per acre annually would approximately meet the nitrogen requirement of the average wheat crop in New South Wales, and under conditions where such a gain could take place regularly, wheat cultivation could, so far as nitrogen is concerned, go on indefinitely. Since any attempt to detect the gain of such a quantity of nitrogen under field conditions would obviously be futile, the present work has been carried out purely under laboratory conditions. Firstly, a survey of the occurrence of *Azotobacter* and nitrogen-fixing clostridia in a number of Australian soils was made, and several representative strains of *Azotobacter* were tested in respect of their nitrogen-fixing capacity.

Secondly, nitrogen fixation in the soil itself was studied by incubating a number of typical wheat soils under varying conditions that should enable organic compounds of the groups mentioned above to become utilized by nitrogen-fixing organisms. The guiding principle in these experiments has been to correlate the changes in nitrogen content found by chemical analysis with the "biological reaction" (Winogradsky, 1925) resulting from the addition of organic compounds to the soil; particular attention was given to the question of the relation between cell counts of *Azotobacter* and quantity of nitrogen fixed. Finally, a series of investigations were made on the production of nitrate and ammonia from the organic ("humus") nitrogen in a number of soils, chiefly from the wheat belt.

(*a*). *Methods.*

Total nitrogen in soil was determined by the Kjeldahl method. At first (soils No. 1–15 in Table 2) the usual method of digestion with concentrated acid was used: finely ground air-dry soil was heated gently for 15–20 minutes with concentrated H_2SO_4 containing 4% salicylic acid to fix the nitrate; after reduction with sodium thiosulphate or zinc powder, potassium sulphate and copper sulphate were added, the mixture was heated until all dark colour had disappeared, and then boiled gently for another 3 hours. It was not until this time that the importance of using dilute sulphuric acid was realized (Bal, 1925; Sreenivasan, 1932). Subsequently a modification of the method of Olsen (1927) was used. Most of the soils to be analysed were very rich in ferric compounds, and it was therefore desirable to avoid the use of up to 5 gm. of reduced iron for reduction of the nitrate, as recommended by Olsen, since the very bulky precipitate resulting therefrom interferes badly with distillation. It was found that zinc powder could be used instead of iron for reduction of the comparatively small quantities of nitrate present in the soils examined here. A series of control analyses of KNO_3-solutions of known concentration showed that quantities of 0·98 to 1·40 mgm. NO_3–N could, with the procedure described below, be recovered with an accuracy of 95–100%, which is close to the titration error; larger amounts—2·80 mgm.—gave significant losses (about 15%). Also when added to nitrate-free soil, quantities of 1·25 mgm. NO_3–N (91·2 parts per million of soil) could be recovered with an accuracy of 95·3 $\pm$ 1·32%. Since the quantity of NO_3–N in the amount of soil used for each analysis never exceeded 0·8 mgm. (usually much less), the method may safely be regarded as satisfactory for the recovery of nitrate within the limits obtaining in the present experiments, although zinc-reduction is unsuitable in case of soils very rich in nitrate (cf. Olsen, 1927, and Sreenivasan, 1935).

The procedure of analysis was as follows: 4 to 20 gm. of soil, depending on its humus content, were placed in a 300 c.c. Kjeldahl flask, and 1 gm. of zinc powder, 5 gm. of K_2SO_4, 20–25 c.c. water and 20 c.c. conc. H_2SO_4 were added. After standing for a few minutes, with repeated shaking, the mixture was boiled slowly for 10–15 minutes, 0·5 gm. $CuSO_4$ was added (if this is added together with the zinc, there results a violent evolution of gas, accompanied by a loss of nitrogen, even with small concentrations of nitrate), and the heating was increased until the water had boiled away and the actual digestion started; if the soil is rich in organic matter, it is preferable not to add the potassium sulphate until the foaming is over. Heating was continued until all dark colour had gone, and then for 3 hours more, during which time the acid was kept slowly boiling (if this is not observed, too low results are obtained, as well as if the heating is

discontinued earlier). After cooling, the mixture was diluted with about 100 c.c. of distilled water and boiled for a few minutes in order to break up any cement-like material. The contents of the digestion flask were then by repeated washings (4–6 times) transferred to a distillation flask, leaving the sand behind in the Kjeldahl flask; a small excess of 20% NaOH was added, and ammonia was distilled off; 28/n H_2SO_4 and NaOH were used for the titration, with methyl red as an indicator. Before titration, the acid was boiled to expel the carbon dioxide and cooled under protection from the atmosphere A blank determination of nitrogen in the reagents was of course subtracted from the titration results. Usually 3 or 4 parallel determinations were made, 5 or 6 in cases of less satisfactory agreement. Extra determinations by this method were done on those soils that had previously been analysed by the "dry" digestion method; no significant differences were found, so that in these soils the "dry" digestion had given correct values (cf. Olsen, 1937). Yet the "wet" digestion is to be preferred, firstly because the digestion proceeds much more quickly, and a better agreement between the parallels is usually obtained (cf. Sreenivasan, 1932), and secondly because soils actually do exist in which the "dry" digestion gives too low results even if the heating is continued for 3 hours after clearing. This was the case with a heavy black basaltic loam (No. 17, Table 2), the type of soil which, according to Bal (1925), is most liable to give low results with dry digestion. The following percentages of nitrogen in dry soil were found:

By "dry" digestion:	By "wet" digestion:
(*a*) 0·1606%	(*a*) 0·1876%
(*b*) 0·1626%	(*b*) 0·1885%
(*c*) 0·1617%	(*c*) 0·1911%
Average: 0·1616%	0·1891%

The sediment was dark and coarsely granular after the dry, but light grey and finely divided after the wet digestion. Although Olsen (1937) found no difference by the two methods in the soils examined by him, there can be no doubt that the addition of water is a necessary precaution, as was also pointed out by Ashton (1936), owing to the ever-present possibility of encountering a soil like the one mentioned.

In some series of experiments, copper sulphate was replaced by 0·2 gm. of selenium, as recommended by Ashton (1936). This proved to be a most efficient catalyst which gave complete clearing of the mixture 20–30 minutes after the start of the actual digestion. Mercury was also tried, but was found to give no higher results than copper sulphate with wet digestion and heating for 3 hours after clearing; its use was therefore given up.

Trials were also made with the Davisson-Parsons method, in which the nitrate is first reduced with Devarda's alloy in alkaline solution (the method of Sreenivasan (1935) is a modification hereof). This method, besides being rather cumbersome, gave no higher results than the acid-reduction method, and was therefore abandoned.

Nitrogen determinations in sand or sand-kaolin mixtures were made as in soil, but on 20–40 gm. of material. These materials, as well as sandy soils, are best weighed off for analysis in a moist condition in order to avoid segregation (cf. Olsen, 1927).

Nitrogen in solution cultures of *Azotobacter* and other microorganisms was determined by the same method, but with only half the quantity of reagents.

A general survey of the agreement between the parallel determinations of nitrogen in soil is given in Figure 1. The coefficients of variability (standard deviation in per cent. of mean of parallel determinations) in 283 nitrogen determinations in soil are here arranged in groups of 0·25%. (The summary does not include the data in Table 8 from analyses with short digestion time, nor the analyses of sand and sand-kaolin mixtures.) The distribution of the frequencies around the mean (1·23%) is strikingly skew. There are cases of serious disagreement corresponding to a standard deviation of ± 4%, but these are quite exceptional; in no less than 194 cases (68·5% of total) we have a standard deviation less than ± 1·5%, which with only 3 parallel determinations corresponds to a mean error less than ± 0·87.

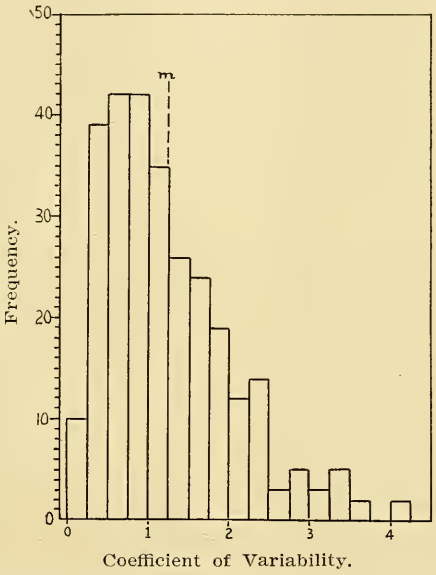

Fig. 1.—Distribution of coefficients of variability in 283 determinations of nitrogen in soil.

Nitrate in soil was determined in a water-extract obtained by leaching 25–75 gm. of air-dry soil with distilled water on a Buchner funnel. The extract was boiled slowly for 1–2 hours with NaOH and $KMnO_4$, the excess of permanganate reduced with a few drops of alcohol, and nitrate was determined by distillation with 1 gm. of Devarda's alloy.

Ammonia was determined by the method of Bengtsson: repeated extraction of a similar quantity of air-dry soil with 0·5 m. KCl, and distillation with MgO. Methyl red and 28/n H_2SO_4 and NaOH were used for the titrations.

(It has sometimes been stated—e.g. Richardson, 1938—that determinations of nitrate and ammonia in soil should be carried out without previous air-drying, which tends to increase the amounts. In view of the recent findings by Waksman and Madhok (1937) this increase might, however, seem to be apparent rather than real so far as nitrate is concerned, and nitrate was the chief form of inorganic soil nitrogen in the present experiments, where the amount of NH_4–N rarely

exceeded 6 parts per million of soil. No serious error need therefore be expected from the air-drying, which greatly facilitated the extraction.)

Determinations of nitrate and ammonia were carried out in duplicate, except in a few cases where there was only sufficient material for one determination. Before the actual determination, qualitative tests were made by extracting 5 gm. of soil with 5 c.c. 0·5 m. KCl and testing a few drops of the filtrate with diphenylamine-sulphuric acid for nitrate (including nitrite, for which no special test was made) and with Nessler's solution for ammonia. If no visible reaction was observed, the quantitative determination was omitted.

All figures for total, nitrate and ammonia nitrogen are in the subsequent tables expressed in terms of parts per million of soil dried to constant weight at 98° C. (abbr. "p.p.m.").

pH-determinations were made colorimetrically by the drop-ratio method of Gillespie on soil extracts obtained by centrifugation of a suspension of air-dry soil in distilled water in the ratio 1:2. Comparatively few soils were so acid as to come below the useful range of brom-cresol-purple (pH 5·5). In these cases it was possible to use methyl red which did not undergo any visible decolourization within the short time necessary to take the readings. Electrometric measurements by means of the quinhydrone electrode were tried, but gave in many instances obviously erroneous results, probably because of the presence of active manganese.

For *counts of Azotobacter* a silica-gel medium with mannite was first tried, as prescribed by Winogradsky (1925–26), but this was later abandoned in favour of an agar medium of the following composition: dextrine 10·0 gm.; K_2HPO_4 0·5 gm.; $MgSO_4$ 0·2 gm.; $FeCl_3$ 0·05 gm.; Na_2MoO_4 0·025 gm.; $CaCO_3$ 5·0 gm.; agar 20·0 gm.; H_2O 1000 c.c. This medium, while not giving such a luxuriant and uncontaminated growth of *Azotobacter* as the silica-gel medium, has the advantages of being less laborious to prepare, easy to sterilize, and of a much firmer texture. For counting purposes there seems to be little to choose between agar and silica-gel media, as found by Curie (1931), de' Rossi (1932*a*), and Turk (1936). Plates for counting were prepared by the method first introduced by Beijerinck (1921) and later used by de' Rossi (1932*a–b*): 4 to 15 gm. of soil were shaken for 3 minutes with sufficient sterile tap water to give a suspension of an initial dilution of 1:3 to 1:10 (higher dilutions were prepared for soils where the growth of *Azotobacter* had been stimulated by addition of organic matter), and portions of 0·2 or 0·4 c.c. of suspension were transferred to usually 3 or 5 parallel plates of sterile dextrine agar in ordinary 10 cm. Petri dishes. The inoculum was spread out as evenly as possible over the surface of agar by means of a stout L-shaped platinum wire, and the excess of water was allowed to evaporate before the dish was closed.[10] The plates were incubated at 28–30° C.; *Azotobacter*-colonies were usually counted after 5 days, but plates on which they did not appear were incubated for at least one week before being discarded; no colonies were ever seen to appear after still longer incubation. The identification of the *Azotobacter*-colonies was greatly facilitated by the circumstance that practically the only species encountered was *Az. chroococcum*, whose colonies on this medium were large, dense and easily distinguishable and showed the characteristic dark pigment after 3 to 4 days (cf. Omeliansky and Ssewerowa, 1911). Plate i, figures 1 and 2, show the appearance of two typical plates.

[10] This is necessary in order to avoid confluent growth of *Azotobacter*. Repeated tests with exposure of sterile plates to the laboratory air for 10-15 minutes showed no contamination with *Azotobacter*.

The reliability of the plate counts of *Azotobacter* was tested by calculating the index of dispersion (Fisher, 1930):

$$\chi^2 = \frac{S(x - \bar{x})^2}{\bar{x}},$$

where x represents the number of colonies per plate, $\bar{x}$ the mean, and $S(x - \bar{x})^2$ the sum of squares of deviations from the mean. If the colony counts give a reliable picture of the density of organisms in the suspension, the values of χ^2 should be distributed with a known frequency (Fisher, 1930, Table III) round a mean value equal to the number of parallel plates minus one. The result of this calculation on the first 100 3-plate counts and the first 70 5-plate counts (not including the many instances where only one or two *Azotobacter*-colonies were found on 3 or 5 parallel plates) is shown in Table 1.

TABLE 1.

Distribution of Values of χ^2 in Plate Counts of Azotobacter.

100 3-plate Sets. (n=2)			70 5-plate Sets. (n=4)		
χ^2	Frequency.		χ^2	Frequency.	
	Expected.	Observed.		Expected.	Observed.
0			0		
	1	3		0·7	1
0·0201			0·297		
	1	0		0·7	0
0·0404			0·429		
	3	2		2·1	1
0·103			0·711		
	5	5		3·5	3
0·211			1·064		
	10	9		7·0	8
0·446			1·649		
	10	13		7·0	5
0·713			2·195		
	20	12		14·0	11
1·386			3·357		
	20	20		14·0	17
2·408			4·878		
	19	8		7·0	12
3·219			5·989		
	10	13		7·0	6
4·605			7·779		
	5	9		3·5	1
5·991			9·488		
	3	3		2·1	4
7·824			11·668		
	1	1		0·7	1
9·210			13·277		
	1	2		0·7	0
	100	100		70	70

There is here in both sets a good agreement between observation and expectation. If anything, there is a tendency to excess among the higher values, but obviously excessive values of χ^2 were only found in one or two instances. The

method must therefore be regarded as satisfactory for determination of the number of *Azotobacter*-cells (or cell-aggregates) capable of developing into colonies on the agar medium.

The same medium was used for isolation and maintenance of pure cultures of *Azotobacter*.

Qualitative tests for presence of Azotobacter were made by inoculating 5 gm. of soil into 50 c.c. of sterile Beijerinck's mannite solution (2% mannite and 0·02% K_2HPO_4 in tap water) in 300 c.c. Erlenmeyer flasks, with addition of about 0·5 gm. $CaCO_3$. The flasks were incubated at 28–30°C. for at least one week and watched for appearance of the characteristic *Azotobacter*-pellicle. In cases of doubtful pellicle formation the surface scum was examined microscopically for presence of *Azotobacter* and a loopful of it streaked out on dextrine agar; this, however, was quite rare. The solution method seems in recent years to have come into disrepute, partly through the criticism of Winogradsky (1925–26), and the view has also before been expressed (e.g. Gainey, 1923) that a large number of *Azotobacter*-cells in the inoculum may be required in order to induce pellicle formation. Few attempts, however, have been made to compare the results of the solution method with those obtained by the methods introduced by Winogradsky. Düggeli (1924) and Wenzl (1934) made counts of *Azotobacter* both on agar plates and by dilution in mannite solution; they found that the solution method gave counts as high as, or even higher than, the plate method. Beck (1935) compared the silica-gel plate and the solution method (on 9 soils only) and found typical pellicles developing only from those soils that also showed *Azotobacter* on the plates.

In the present investigation there were 102 simultaneous plate counts and solution tests carried out. They showed the following agreement:

		Presence of *Azotobacter* by solution test.		
		Positive.	Negative.	Total.
Presence of *Azotobacter* by plate count.	Positive	30	5	35
	Negative	18	49	67
	Total	48	54	102

In 79 cases (77·4%) the two methods have shown agreement. In the remaining 23 cases, the solution method has shown presence of *Azotobacter* more than 3 times as frequently as the plate method. In all cases where the plate method gave a count of 10 or more *Azotobacter*-colonies per gm. of soil, a more or less typical pellicle developed in the mannite solution. In the 5 cases where the plate method gave positive and the solution method negative results, the plate counts indicated only 3–5 *Azotobacter*-colonies per gm. of soil. Unless very large plates inoculated with 1 to 2 gm. of soil be used, the solution method thus seems actually better adapted for detecting a sporadic presence of *Azotobacter*, and there is no reason to think that the results found by means of this method by earlier investigators (e.g. Christensen, 1915; Gainey, 1923) give a false picture of the distribution of *Azotobacter* in soil, as suspected by Winogradsky (1926). Yet it is desirable to combine it with other methods, not least in view of the discovery by Smith (1935) of *Azotobacter*-strains incapable of utilizing mannite.

Tests for presence of anaerobic nitrogen-fixing bacteria (butyric acid bacilli) were made in a modified Winogradsky's glucose solution, containing 10·0 gm. glucose, 0·5 gm. K_2HPO_4, 0·2 gm. $MgSO_4$, 0·2 gm. NaCl, 0·02 gm. $FeCl_3$, 0·01 gm. Na_2MoO_4, 10·0 gm. $CaCO_3$, and 1·0 gm. agar in 1000 c.c. H_2O. The medium was filled in ordinary test tubes to a height of 10 cm., sterilized, inoculated with soil

suspension corresponding to 0·1 gm. of soil, and incubated at 28–30°C. When gas formation appeared, the sediment was examined microscopically for presence of clostridia staining blue with Lugol's iodine solution. No attempt was made to count these organisms by cultural methods, in view of the extremely low percentage of the actual number of individuals that may be capable of reproduction in artificial media (Dorner, 1924).

Direct microscopic counts of total numbers of bacteria in soil were carried out by the indigo method of Thornton and Gray (1934). Rose bengale was used for staining instead of erythrosine. The counts were usually made in 50 microscopic fields from 10 drops of soil suspension of a dilution of 1:4 to 1:10. Separate counts were made of *Azotobacter*- and *Clostridium*-like organisms which, however, only rarely were so numerous that they could be counted with any reasonable accuracy (standard error less than ± 30%). The standard percentage error of the counts was estimated by the formula of Fisher:

$$\% \text{ S.E.} = 100\sqrt{\frac{1}{I} + \frac{1}{B}} \qquad \text{(Thornton and Gray, 1934)}$$

where I and B represent the total numbers counted of indigo particles and bacteria, respectively.

All numbers of microorganisms are expressed on the basis of soil dried at 98°C.

(*b*). *On the Occurrence of Nitrogen-fixing Bacteria in Australian Soils.*

A large amount of research has been done on the distribution of *Azotobacter* in soils from nearly all geographical regions (for references, see Waksman, 1932, and de' Rossi, 1932*c*). The earlier qualitative tests by means of the solution method have shown that the soil reaction is a factor of prime importance in controlling the distribution of *Azotobacter* in soil (Christensen, 1915). Further studies by Gainey (1923) and numerous later investigators have shown that a certain critical point exists at pH 6·0, above which occurrence of *Azotobacter* is common and below which it is rare. (The same pH-value, as shown by Burk, Lineweaver and Horner (1934), represents the limit of acidity for functioning of the nitrogen-fixing enzymic complex.) Yet the occurrence of *Azotobacter* in soils considerably more acid than pH 6·0 is by no means excluded (Gainey, 1923; Vandecaveye and Anderson, 1934, and others). Loew (1927) mentions a frequent occurrence of *Azotobacter* in tropical soils of acid reaction (pH not stated). Altson (1935) reported the isolation, from acid soil in Malaya, of a species of *Azotobacter* that seemed sensitive to $CaCO_3$ and capable of nitrogen fixation in media of pH 4·8–5·0; unfortunately the study of this remarkable organism was far from complete. A similar (identical?) organism has recently been described by Starkey and De (1939). Apart from certain observations by Jones and Murdoch (1919), Beijerinck (1921) and Düggeli (1924), few attempts were made to estimate the actual numbers of *Azotobacter* in soil, until Winogradsky (1925) introduced new methods, consisting partly in microscopic examination of the soil and partly in "spontaneous cultures" in selective media. On the basis of tests on silica-gel medium, Winogradsky (1926) distinguished three categories of soil in respect of nitrogen-fixing capacity: "active" soils, showing development of 2000–3000 *Azotobacter*-colonies per gm. of soil; "less active" soils, showing a variable but smaller number, and inactive soils, not showing development of *Azotobacter*. The maximal number of colonies reported by Winogradsky (1928) was 12,600 per gm. Similar tests on silicic-acid or agar media by other authors (Curie, 1931.

Vandecaveye and Anderson, 1934, and Turk, 1936, in U.S.A., Lochhead and Thexton, 1936, in Canada, Ziemecka, 1932, in England, de' Rossi, 1932*b*, in Italy, Wenzl, 1934, in Austria, De and Pain, 1936, in India) have given results in agreement herewith: like Jones and Murdoch (1919) and Beijerinck (1921), these authors found that the maximal numbers of *Azotobacter* rarely exceed a few thousand per gm. of soil and often reach only a few hundreds. It is common experience that nitrogenous fertilizers tend to reduce the numbers of *Azotobacter* (Düggeli, 1924; Winogradsky, 1928; Ziemecka, 1932; Lochhead and Thexton, 1936), but a rich *Azotobacter*-flora is usually assumed to be associated with generally high soil fertility (Remy, 1909; Beijerinck, 1921). Comparatively high numbers (up to 21,000 per gm.) were found by de' Rossi (1932*b*) who, unlike most other investigators, used soil suspension instead of the soil itself as an inoculum for the plates; some aggregates of *Azotobacter*-cells may hereby be broken up and the colony-count increased.[11] In agreement herewith, similar high numbers (15,000 or more per gm.) were reported by Düggeli (1924) and Burgess (1929), who used the method of dilution in liquid media. Besides these, some extraordinary numbers have now and again been reported. Dhar and Seshacharyulu (1936) found 1·3 to 2·8 mill. *Azotobacter* per gm. by plate counts in soil from plots *not* treated with molasses, and Kostytchev (1924) mentions a Crimean tobacco soil with more than 10 mill. *Azotobacter* per gm. In the first case it seems uncertain whether all the colonies counted were really *Azotobacter*, and in the second it is not stated whether cultural or microscopic methods were used. Several Russian investigators quoted by Dianowa and Woroschilowa (1931) claim to have found similar or even much higher numbers of *Azotobacter* by microscopic methods, but these findings, as Dianowa and Woroschilowa point out, are inconclusive, since other bacteria may produce cell types morphologically indistinguishable from *Azotobacter*.[12]

Anaerobic nitrogen-fixing bacteria of the butyric acid bacilli group are almost constant soil inhabitants, as first shown by Bredemann (1909*b*), and their numbers as indicated by cultural methods are generally higher than those of *Azotobacter* (de' Rossi, 1932*c*; Willis, 1934).

Few publications have yet appeared on the occurrence of nitrogen-fixing bacteria in Australian soils. Darnell-Smith (1912) reported the isolation of *Azotobacter* from three soils from New South Wales. Lewcock (1925) stated that he found *Azotobacter* constantly by the solution method in a not specified number of soils from South Australia, and thought that stimulation of their activity by phosphatic fertilizers was the cause of the general inefficiency of nitrogenous fertilizers. Beck (1935) arrived at entirely different results; by the use of silica-gel plates with mannite (sometimes combined with solution tests) he found *Azotobacter* in only 10 (all of pH 6·9 to 7·4) out of 33 South Australian soils; the maximal colony count was 560 per gm. It is to be noted that all the samples had been stored from 1 week to 8 months between sampling and testing. Penman and Rountree (1932), using the same technique, found *Azotobacter* in numbers of 50 to 260 per gm. in an approximately neutral Victorian soil under wheat or fallow, in which latter case it generally seemed more abundant. The authors concluded without any direct experimental evidence, that the vigorous nitrate production during fallowing was due to the combined action of *Azotobacter* and nitrifying bacteria. Swaby (1939) found *Azotobacter* present in 21 out of 80

[11] Swaby (1939), however, found only small increases in colony numbers, when soil suspension was used as an inoculum instead of the undisturbed soil.

[12] Winogradsky (1925) and Rossi et al. (1936) make the same reservation.

Victorian soils of pH 4·1–8·8, by plate counting on glucose agar. The highest number was 1600 per gm., and only 3 soils of pH 7·0–8·8 contained more than 100 per gm.; very few soils of pH 6·0 or less contained *Azotobacter* at all. *Clostridium butyricum* was found constantly in 57 soils, in numbers up to 1640 per gm., but mainly represented by spores.

The present investigations were carried out on 85 soil samples, mostly from the wheat district of New South Wales. A general description of the soils from which the samples were taken is found in Table 2. The geographical distribution of these soils is the following:

From New South Wales (65 samples):

Within the wheat belt (55 samples):

Northern Tableland and North-Western Slope: Nos. 16–18, 64–67, 80–82 (10 samples).

Central Tableland: Nos. 10, 11, 45–48, 57, 58, 72, 73 (10 samples).

Central Plain: Nos. 6, 7, 70, 71 (4 samples).

Central Western Slope: Nos. 53, 54, 74, 75, 78, 79 (6 samples).

South-Western Slope: Nos. 1, 2, 12–15, 19–21, 35, 38–42, 51, 52, 63, 76, 77 (20 samples).

Riverina: Nos. 49, 50, 83–85 (5 samples).

Outside the wheat belt (10 samples): Nos. 3–5, 8, 9, 44, 55, 56, 59, 60.

From Victoria (15 samples, all within the wheat belt): Nos. 22–30, 36, 37, 61, 62, 69, 70.

From Queensland (5 samples):

Within the wheat belt: Nos. 31–34.

Outside the wheat belt: No. 43.

The approximate location of the samples is shown on Text-figure 2.

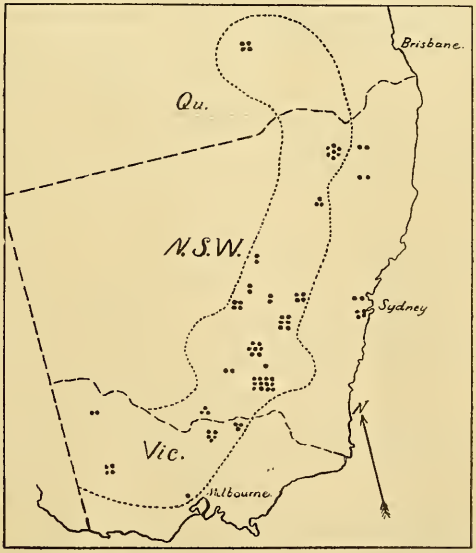

Fig. 2.—Map showing approximate location of soil samples (except No. 43, in Queensland). Broken lines: State boundaries. Dotted line: approximate boundaries of the wheat belt.

TABLE 2.

General Description of Soils Examined. Occurrence of N-fixing Bacteria.
(Soils marked * are from localities outside the wheat belt.)

Journal Number and General Character of Soil.	H_2O %.	pH.	*Azotobacter.* In Mannite† Solution.	*Azotobacter.* By Plate Count.	Clostridia in Glucose Solution.
1.—Red loam, good wheat field. Wagga, N.S.W. Sampled Nov., 1934, examined Jan., 1936 ..	(air-dry)	6·0	−		+
2.—Grey-brown loam, poorer wheat field. Same locality. Sampled Nov., 1934, examined Jan., 1936	(air-dry)	6·5	−		+
*3.—Coarse dark sand, uncultivated. Rose Bay North, Sydney. Sampled Feb., 1935, examined Jan., 1936	(air-dry)	4·8	−		
*4.—Heavy red loam, rich in humus. Lucerne field, northern tablelands, N.S.W. Sampled Dec., 1934, examined Jan., 1936	(air-dry)	5·5	−		
*5.—Heavy red loam, Same character and locality as No. 4. Sampled Dec., 1934, examined Jan., 1936	(air-dry)	4·7	−		
6.—Red loam, wheat stubble. Condobolin Exp. Farm, N.S.W. Receiving 156 lb. super. per annum. Sampled 18/1/36, examined 23/1/36..		5·6	−		
7.—Same soil type and locality as No. 6. Wheat stubble, unfertilized. Sampled 18/1/36, examined 23/1/36		5·1	−		
*8.—Grey sand mixed with clay, uncultivated. University of Sydney. Sampled and examined 11/2/36		7·4	+		
Do. after drying				9	
*9.—Black loam, rich in humus, under grass. University of Sydney. Sampled and examined (*a*) 7/3/36		6·9	+		
Do. after drying				590	
(*b*) 6/4/37	17·9	6·6	+	2360	
10.—Grey-brown sandy loam, wheat stubble. Cowra Exp. Farm, N.S.W. Receiving 168 lb. super. per annum. Sampled 16/3/36, examined 18/3/36		6·4	(+)		
Do. after drying			+	20	
11.—Same soil type and locality as No. 10. Wheat stubble, unfertilized. Sampled 16/3/36, examined 18/3/36		6·5	(+)		
Do. after drying			+	0	
12.—Yellowish-red loam, lucerne field (wheat until 1930). Wagga Exp. Farm, N.S.W. Receiving 112 lb. super. per annum. Sampled 30/3/36, examined 1/4/36		5·7	+		
Do. after drying			+	67	
13.—Same soil type and locality as No. 12. Unfertilized lucerne plot. Sampled 30/3/36, examined 1/4/36		6·1	−		
Do. after drying			+	0	
14.—Yellowish-red loam, fallow after oats. Temora Exp. Farm, N.S.W. Receiving 168 lb. super. per crop. Sampled 14/4/36, examined 17/4/36..		5·6	+		
Do. after drying			−	0	

† −, no pellicle ; +, pellicle formation ; (+), no real pellicle, but presence of *Azotobacter* revealed by microscopic examination.

15.—Same soil type and locality. Fallow, unfertilized. Sampled 14/4/36, examined 17/4/36		5·4	+
Do. after drying			− 0
16.—Heavy brown loam, wheat field. Swan Vale, Inverell, N.S.W. Yield in 1935, 15 bus. Sampled 4/6/36, examined 8/6/36		7·1	(+)
Do. after drying			(+) 0
17.—Heavy black loam, wheat field. Little Plain, Inverell, N.S.W. Yield in 1935, 36 bus. Sampled 3/6/36, examined 8/6/36		6·2	−
Do. after drying			0
18.—Heavy red-brown loam, wheat field. Little Plain, Inverell, N.S.W. Yield in 1935, 37 bus. Sampled 3/6/36, examined 8/6/36		6·1	−
Do. after drying			+ 7
19.—Red sandy loam, wheat field. Junee, N.S.W. Cultivated 50 years. Av. yield, 26 bus. Sampled 8/6/36, examined 12/6/36		6·0	−
20.—Red light loam, wheat field. Temora, N.S.W. Rotation wheat, fallow. Yield since 1929, 24–33 bus. Sampled 9/7/36, examined 14/7/36		6·0	− 0
21.—Red light loam, wheat field. Temora, N.S.W. Rotation wheat, oats, fallow. Yield of wheat since 1929, 18–35 bus. Sampled 9/7/36, examined 14/7/36		5·9	− 0
22.—Fine grey loam, fallow after wheat. Werribee Exp. Farm, Vic. Sampled 3/7/36, examined 6/7/36		5·8	−
23.—Red-brown sand, wheat stubble. Mallee Research Station, Walpeup, Vic. Cleared 1933. Yield of wheat, 1934, 8·5 bus. Sampled 15/7/37, examined 17/7/37		7·6	+ 0
24.—Red sand, poor in humus, wheat stubble. Same locality as No. 23. Yield of wheat in 1935, 27·5 bus. Sampled 15/7/36, examined 17/7/36		7·2	+
25.—Yellowish-grey loam, wheat stubble. Rutherglen Exp. Farm, Vic. Cultivated 20 years. Fallow, wheat (or oats). Yield of wheat, 1935, 15 bus. Sampled 21/7/36, examined 23/7/36.		6·5	+
Do. after drying			− 0
26.—Heavy black loam, wheat stubble. Dookie Agr. College, Vic. Cultivated 50 years. Yield, 1935, 35 bus. Sampled 30/7/36, examined 4/8/36		7·5	−
Do. after drying			(+) 7
27.—Fine red-grey loam, wheat stubble. Same locality. Cultivated 45 years. Yield 1935, 33 bus. Sampled 30/7/36, examined 4/8/36		5·8	−
28.—Dark red loam, wheat stubble. Same locality. Cultivated 45 years. Yield 1935, 39 bus. Sampled 30/7/36, examined 4/8/36		5·7	−
29.—Heavy grey loam, stubble pasture after wheat. Longerenong Agr. College, Vic. Cultivated 40 years. Wheat, stubble, pasture, fallow. Yield of wheat, 14–42 bus. Sampled 7/8/36, examined 25/8/36		7·6	(+) 0
30.—Same soil type and locality as No. 29. Under wheat crop. Cultivated 40 years. Same rotation and yield. Sampled 7/8/36, examined 25/8/36		7·7	(+)
31.—Red-brown sand, poor in humus. Roma State Farm Reservation, Q. Vines and citrus fruit 20 years. Sampled 15/2/37, examined 25/2/37	(air-dry)	6·5	+ 0

TABLE 2.—*Continued.*

Journal Number and General Character of Soil.	H_2O %.	pH.	*Azotobacter.* In Mannite† Solution.	*Azotobacter.* By Plate Count.	Clostridia in Glucose Solution.
32.—Red-brown sand, poor in humus, wheat stubble. Same locality as No. 31. Cultivated 20 years. Av. yield of wheat, 15·9 bus. Sampled 15/2/37, examined 25/2/37	(air-dry)	6·1	−	0	
33.—Red-brown sand, poor in humus, wheat stubble. Near localities No. 31 and 32. Sampled 15/2/37, examined 25/2/37	(air-dry)	6·2	+	0	
34.—Heavy black loam, pasture, cropped with wheat a few times last 8 years. Close to localities No. 31–33. Sampled 15/2/37, examined 25/2/37	(air-dry)	7·6	+	7	
35.—Red loam, wheat stubble. Wagga Exp. Farm, N.S.W. Sampled 28/4/37, examined 3/5/37	11·1	6·0	−	0	
36.—Yellowish-red loam, pasture. Rutherglen Exp. Farm, Vic. (wheat-oats-fallow until 1927). Sampled 29/4/39, examined 3/5/39	8·0	5·5	−	0	
37.—Yellowish-red loam, fallow after wheat. Same locality as No. 36. Yield of wheat 1936, 9 bus. Sampled 29/4/37, examined 3/5/37	4·5	5·6	−	0	
38.—Red loam, pasture. Wagga Exp. Farm, N.S.W. Sampled 26/5/37, examined 27/5/37	7·2	5·9	−	0	
39.—Yellowish-red loam. Same locality as No. 38. Lucerne field. Sampled 26/5/37, examined 27/5/37	6·6	5·7	−	0	
40.—Red loam, pasture. Same locality. Sampled 26/5/37, examined 27/5/37	6·6	6·5	−	0	
41.—Red loam, lucerne field. Same locality. Sampled 3/6/37, examined 9/6/37	11·8	5·9	−	0	+
42.—Red loam, stubble after wheat for hay in 1936. Same locality. Sampled 3/6/37, examined 9/6/37	13·0	6·0	−	0	+
*43.—Black loam, rich in humus, cotton field. Biloela, Q. Sampled 5/2/37, examined 5/4/37	6·6	7·3	+	740	
*44.—Heavy grey loam, experimental plots. School of Agriculture, Univ. of Sydney. Fallow; crop of field peas ploughed in on day of sampling. Sampled and examined 9/4/37	10·6	7·4	+	1640	+
45.—Red-brown sandy loam, under wheat. Bathurst Exp. Farm, N.S.W. (paddock No. 10). Sampled 13/8/37, examined 18/8/37	5·5	5·6	−	0	+
46.—Red-brown sandy loam, under wheat. Same locality as No. 45 (paddock No. 18). Sampled 13/8/37, examined 18/8/37	8·2	5·5	−	0	+
47.—Light brown sandy loam, under wheat. Cowra Exp. Farm, N.S.W. Cultivated 35 years. av. yield: wheat 25 bus., oats 36 bus. Sampled 19/8/37, examined 23/8/37	6·8	6·5	−	5	+
48.—Light brown sandy loam, under wheat. Same locality as No. 47. Cultivated 30 years. Yield similar to No. 47. Sampled 19/8/37, examined 23/8/37	8·0	6·3	+	11	+

† −, no pellicle; +, pellicle formation; (+), no real pellicle, but presence of *Azotobacter* revealed by microscopic examination.

49.—Red loam, fallow. Corobimilla via Narrandera, N.S.W. Cultivated 16 years, wheat, oats, fallow. Yield of wheat, 14–33 bus. Sampled 2/9/37, examined 6/9/37	9·6	5·8	—	6	+
50.—Red loam, fallow. Brobcnah, N.S.W. Cultivated 35 years, wheat, oats, fallow, later wheat, fallow. Av. yield of wheat, 23 bus. Sampled 8/9/37, examined 10/9/37	9·0	6·1	—	0	+
51.—Red loam, under wheat. Temora Exp. Farm, N.S.W. Cultivated 8 years, wheat, fallow. Av. yield, 27 bus. Sampled 10/9/37, examined 14/9/37	10·9	6·6	—	0	+
52.—Another sample from the same field as No. 51. Sampled 10/9/37, examined 14/9/37	10·4	6·3	—	0	+
53.—Red loam, under wheat. Trangie Exp. Farm, N.S.W. Cultivated 20 years wheat, fallow, since 1929 wheat annually; yield 8–25 bus. Sampled 15/9/37, examined 16/9/37 ..	(air-dry)	6·7	—	0	+
54.—Another sample from the same field as No. 53. Sampled and examined same time	(air-dry)	6·8	—	0	+
*55.—Heavy brown loam, under wheat. New England Exp. Farm, N.S.W. Cultivated about 20 years. Sampled 23/9/37, examined 24/9/37..	12·5	5·7	—	0	+
*56.—Another sample from the same field as No. 55. Sampled and examined at the same time ..	12·7	5·7	—	0	+
57.—Light grey-brown sandy loam, under wheat. Bathurst Exp. Farm, N.S.W. Sample included wheat roots. Sampled 30/9/37, examined 1/10/37.					
Soil	4·5	5·5	—	0	+
Roots	17·0			0	
58.—Same soil type and locality as No. 57. Another field under wheat. Sample included wheat roots. Sampled 30/9/37, examined 2/10/37.					
Soil	4·9	5·5	—	0	+
Roots	20·0			0	
*59.—Fine grey sandy loam, under wheat for hay. Hawkesbury Agr. College, Richmond, N.S.W. Cultivated 40 years, cereals annually since 1930. Sample included wheat roots. Sampled 7/10/37, examined 8/10/37.					
Soil	(air-dry)	5·4	—	0	+
Roots				0	
*60.—Same soil type and locality as No. 59, under wheat for hay. Cultivated 35 years, cereals annually since 1924. Sample included wheat roots. Sampled 7/10/37, examined 8/10/37.					
Soil	(air-dry)	5·2	—	0	+
Roots				0	
61.—Heavy grey loam, under wheat crop. Longerenong Agr. College, Vic. Cultivated 40 years, mainly wheat, oats, fallow, since 1927. Yield of wheat, up to 53 bus. Sampled 5/10/37, examined 9/10/37	15·8	8·3	—	0	+
62.—Same soil type and locality as No. 61, under wheat crop. Cultivated 40 years, mainly wheat, pasture, fallow since 1927. Yield of wheat, 12–42 bus. Sampled 5/10/37, examined 9/10/37	16·5	8·3	—	0	+
63.—Yellowish-red loam, under wheat. Temora Exp. Farm, N.S.W. Since 1923, mainly wheat, fallow. Av. yield, 24 bus. Sample included wheat roots. Sampled 7/10/37, examined 11/10/37					
Soil	(air-dry)	5·7	+	0	+
Roots				0	

TABLE 2.—*Continued.*

Journal Number and General Character of Soil.	H_2O %.	pH.	*Azotobacter.* In Mannite† Solution.	*Azotobacter.* By Plate Count.	Clostridia in Glucose Solution.
64.—Heavy red-brown loam, rich in humus. Swan Vale, Inverell, N.S.W. Under wheat. Cultivated 20 years, wheat annually. Av. yield, 24–27 bus. Sampled 8/10/37, examined 11/10/37	13·9	6·4	–	0	
65.—Heavy black loam, under wheat. Auburn Vale, Inverell, N.S.W. Cultivated 15–20 years, lucerne 1929–35, also wheat annually. Yield never below 30 bus. Sampled 8/10/37, examined 11/10/37	15·6	6·7	–	0	+
66.—Heavy black loam, under wheat. Inverell, N.S.W. Cultivated 15 years, wheat, maize. Yield of wheat, 30–36 bus. Sampled 8/10/37, examined 11/10/37	20·4	6·5	+	6	+
67.—Heavy black loam, under wheat. Close to previous locality. Good yields after long fallow, otherwise poor. Yield of wheat, 15 bus. Sampled 8/10/37, examined 11/10/37	22·5	6·9	–	0	+
68.—Red loam, under wheat. Dookie Agr. College, Vic. Cultivated 50 years, since 1925 wheat, oats, fallow. Yield of wheat, 24–42 bus. Sample included wheat roots. Sampled 6/10/37, examined 12/10/37.					
Soil	9·0	5·7	–	0	+
Roots	17·0			0	
69.—Heavy dark-grey loam, under wheat. Dookie Agr. College, Vic. Cultivated 50 years, wheat, fallow since 1924. Yield 18–42 bus. Sampled 6/10/37, examined 12/10/37	17·7	7·0	–	0	+
70.—Red loam, under wheat. Condobolin Exp. Farm, N.S.W. Cleared 1930, oats 1931, since then wheat, fallow. Yield, 9–19 bus. Sampled 8/10/37, examined 13/10/37	(air-dry) 5·6		–	0	+
71.—Red loam, under wheat. Same locality as No. 70. Old cultivated paddock, since 1930 wheat, fallow. Yield, 7–13 bus. Sampled 8/10/37, examined 13/10/37	(air-dry) 5·7		–	0	+
72.—Coarse sandy yellowish-red loam, under wheat. Cowra Exp. Farm, N.S.W. Cultivated 35 years, wheat, oats, fallow. Av. yield wheat, 25 bus.; oats, 36 bus. Sampled 11/10/37, examined 14/10/37. Sample included wheat roots.					
Soil	5·3	5·8	–	0	+
Roots				0	
73.—Light sandy, red-brown loam, under wheat. Cowra Exp. Farm, N.S.W. Cultivated 40 years, wheat, oats, fallow. Yield similar to No. 72. Sample included wheat roots. Sampled 11/10/37, examined 14/10/37.					
Soil	8·7	6·5	–	0	+
Roots				0	
74.—Heavy greyish-brown loam (typical of the Myall Belt), fallow. Nelungoloo, Parkes, N.S.W. Cultivated, wheat 20 years. Av. yield, 23 bus. Sampled 19/11/37, examined 25/11/37	(air-dry) 7·1		–	0	+

† –, no pellicle; +, pellicle formation; (+), no real pellicle, but presence of *Azotobacter* revealed by microscopic examination.

75.—Red loam, under wheat. St. Evans, Parkes, N.S.W. Cultivated 35 years, wheat, fallow. Yield, 30–36 bus. Sampled 20/11/37, examined 25/11/37	7·5	6·0	—	0	+
76.—Yellowish-red loam, under wheat. Wagga Exp. Farm, N.S.W. Cultivated 15 years, fallow, wheat (hay). Sample included wheat roots. Sampled 10/12/37, examined 13/12/37.					
Soil	(air-	5·9	—	0	+
Roots	dry)			0	
77.—Same soil type and locality as No. 76. Same rotation, crop harvested for grain. Av. yield, 22 bus. Sample included wheat roots. Sampled 10/12/37, examined 13/12/37.					
Soil	(air-	5·7	—	0	+
Roots	dry)			0	
78.—Red loam, wheat field sown after fallow. Trundle, N.S.W. Cropped 40 years, wheat, fallow. Av. yield, 15 bus. Sampled 14/6/38, examined 17/6/38	(air-dry)	5·5	—	0	+
79.—Same soil type and locality as No. 78. Stubble after wheat, 1937. Cultivated 35 years, wheat, fallow. Av. yield, 20 bus. Sampled 14/6/38, examined 17/6/38	4·8	6·3	+	0	?
80.—Red-brown loam, wheat field. Curlewis, Gunnedah, N.S.W. Wheat 3 years, after grazing 10 years. Sampled 20/6/38, examined 22/6/38	10·4	6·9	+	0	?
81.—Heavy brown loam, rich in humus. Locality near No. 80. Stubble after wheat, 1937. Sampled 20/6/38, examined 22/6/38	20·7	8·0	+	960	+
82.—Heavy dark brown loam, rich in humus. wheat field. Locality near Nos. 80 and 81. Cultivated for 3 years. Sampled 4/8/38, examined 8/8/38	22·2	7·9	+	0	+
83.—Red loam, wheat field. Finley, N.S.W. Cultivated 20 years, wheat, oats, fallow. Av. yield of wheat, 15 bus. Sampled 1/9/38, examined 5/9/38	8·3	5·9	—	0	+
84.—Red loam, wheat field. Finley, N.S.W. Yield and rotation similar to No. 83. Sampled 1/9/38, examined 5/9/38	11·0	6·0	—	0	
85.—Red loam. Finley, N.S.W. Fallow. Yield and rotation similar to Nos. 83 and 84. Sampled 1/9/38, examined 5/9/38	9·6	6·6	—	0	—

Soils No. 1 to 5 had been stored for a considerable time in an air-dry condition before examination, and were used only for tentative experiments, the results of which are not strictly comparable with the rest. The other samples were taken straight from the fields, and represent the top 6 inches of soil. Owing to the wide distribution of the localities sampled, only few samples could be taken by myself, and strict aseptic precautions could not be taken in the sampling; however, sterilized tins were provided for the samples where possible. The tests were carried out as soon as possible after the arrival of the samples at the laboratory, usually on the same day; if this could not be done, the samples were stored in a refrigerator until examination. In most cases not more than 5 days elapsed between sampling and microbiological examination.[13] After the tests had been made, the soil was

[13] The longer delay in case of Nos. 29 and 30 was due to misunderstanding concerning the dispatch of the samples, and in case of Nos. 31-34 to the author's illness.

spread out in a thin layer on clean paper, air-dried, ground, sieved through a 20-mesh sieve, and stored for future use.

All soils were tested for *Azotobacter* by the solution method and, after the silica-gel medium had been abandoned, also by plate counts on dextrine agar (3 or 5 parallel plates, dilution 1:10 to 1:25). The tests were repeated on some of the soils after air-drying. Tests for anaerobic nitrogen-fixers were carried out in 45 soils only.

The results of the microbiological examinations are seen in Table 2. *Azotobacter* is of rather infrequent occurrence, being present in only 27, or 32%, of the 85 soils examined (the few soils that showed presence of *Azotobacter* only after air-drying, viz. Nos. 13, 18, and 26, are not considered positive owing to the possibility of contamination). If we consider only the freshly taken samples from the wheat belt (omitting Nos. 1–5, 8, 9, 43, 44, 55, 56, 59, and 60) we have 72 soils, of which 23, or 32%, contain *Azotobacter*. The occurrence of this organism in relation to soil reaction is shown in Figure 3. We find here 42 soils of pH 6·0 and less, of which 5, or 11·9%, contain *Azotobacter*; the most acid soil in which it was found was No. 15, of pH 5·4. Among the 43 soils of pH above 6·0 there are 22, or 50·1%, that contain *Azotobacter*, which seems to have its greatest relative frequency in the pH-interval 7·0 to 8·0. The corresponding figures for the 72 fresh samples from the wheat belt are 14·4% at pH 6·0 and less, and 47·4% above pH 6·0. The graph also shows that a reaction of pH 5·5 to 6·5 is by far the most common in the wheat soils examined here, although of course the prevailing soil reaction in the vast areas of the wheat belt cannot be judged on the basis of these few observations; no systematic work has yet been done on this question in New South Wales.

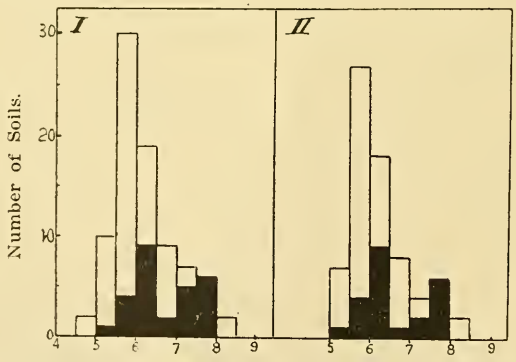

Fig. 3.—Occurrence of *Azotobacter* in relation to soil reaction: I, all samples; II, fresh samples from the wheat belt. (Black part of columns indicates number of samples containing *Azotobacter*.)

Table 2 further shows that *Azotobacter* is usually present in quite small numbers—from 5 to 20 colonies per gm. of soil, or so few that they cannot be detected by counting on the number of parallel plates used here. Only 4 soils (Nos. 9, 43, 44 and 81) are really rich in *Azotobacter*, with numbers of 600 to 2,360 per gm. of soil, and only one of these—No. 81—is a wheat soil. The soils are all of pH 6·6 to 8·0, and are considerably richer in humus than the average.

Finally, no direct correlation can be traced between the general productivity of the soils and the presence or absence of *Azotobacter*. For instance, they are absent or only sporadically present in the Wimmera soils from Victoria (Nos. 29, 30, 61 and 62), which are representatives of the most fertile type of wheat soils in Australia.

Unlike *Azotobacter*, the clostridia were almost constantly present, being found in all the 45 samples examined, with the exception of No. 85, and possibly also Nos. 79 and 80, where some fermentation took place, but where clostridia could not be seen with certainty on microscopic examination.

Generally speaking the results thus agree well with those found in other parts of the world, and also with those of Beck (1935), although in the present series *Azotobacter* is definitely more common in acid soils (Beck's failure to find *Azotobacter* in soils of pH below 6·9 may be due to the use of stored soil samples, since *Azotobacter* tends to die out on storage in acid soils). The agreement with Swaby's (1939) results is excellent.

No other organism than *Azotobacter* ever produced more than a trace of growth on the dextrine agar. Before the *Azotobacter*-colonies became distinguishable, which usually happened after 48 hours, the plates generally showed many small dewdrop-like colonies which soon coalesced into a thin, colourless, mucoid layer over the whole plate; this growth came to a standstill after 3 to 4 days, and was never so conspicuous as to suggest the presence of nitrogen-fixing bacteria (cf. Winogradsky, 1926). The medium thus appears very selective for *Azotobacter*, but this of course does not prove that no other aerobic nitrogen-fixing microorganisms exist in the soil.

In connection with this experimental series we shall mention some observations on the occurrence of *Azotobacter* in the rhizosphere of wheat. The rhizosphere is the zone immediately surrounding the plant roots; it contains a microbial population far more abundant than that of the soil itself, because in this zone there is an extra supply of organic matter represented by decaying root particles and in some cases probably also organic compounds secreted by living roots (see Waksman, 1932). Thom and Humfield (1932) made the important observation that the plant roots exert a kind of buffer effect on the soil, and create an approximately neutral reaction in the rhizosphere and, according to Starkey (1931), the concentration of nitrate is lower here than in the soil itself. All these circumstances together suggest that nitrogen-fixing bacteria, and *Azotobacter* in particular, might find a suitable habitat in the rhizosphere even if the conditions in the actual soil were unfavourable, and that the rhizosphere generally would offer better opportunities for nitrogen fixation; Kostytchev (1924) and Loew (1927) have, chiefly on theoretical grounds, ascribed a great importance to the activity of *Azotobacter* under these conditions and, as mentioned previously, Truffaut and Bezssonoff (1925) and von Caron (1934) claim to have grown maize and barley in sand culture inoculated with *Azotobacter* or other (allegedly) nitrogen-fixing bacteria, which under these conditions presumably could only utilize the organic root secretions and dead root parts. The direct experimental evidence, however, is less positive. Beijerinck (1909), Poschenrieder (1929–30) and Truffaut and Vladykov (1930) observed the common occurrence of *Azotobacter* in the neighbourhood of the roots of different plants (legumes, cruciferous plants, wheat), without giving any numerical data. Lyon and Wilson (1926) grew timothy aseptically in sterilized soil inoculated with *Azotobacter*, and found about twice as high numbers of this organism in planted as in

unplanted soil; the numbers, however, were always abnormally high (several millions per gm.) in comparison with normal soil. Starkey (1929) and Gräf (1930), using the solution method, found no definite evidence of more intensive nitrogen fixation or higher numbers of nitrogen-fixing bacteria in the rhizosphere of various plants, although the general micropopulation was immensely richer here than in the adjacent soil. Also in later experiments, where he used the contact slide method of Rossi and Cholodny for direct microscopical observation, Starkey (1938) found *Azotobacter*-like organisms only sparsely represented among the micropopulation in the rhizosphere of various plants at different stages of growth. Krasilnikov (1934) found the organic root secretions of wheat, produced in aseptic sand culture, altogether unsuitable as food material for *Azotobacter*, while the secretions of maize were of only inferior value; these results might not necessarily apply under natural soil conditions.

In the present series of investigations there were 10 cases (Nos. 57–60, 63, 68, 72, 73, 76, 77) where a considerable amount of roots of still-growing wheat plants was present in the sample. In these cases a separate count of *Azotobacter* was carried out on the root material (5 parallel plates, dil. 1:50). The results, as on the soils themselves, were always negative, as shown in Table 2. If *Azotobacter* thus occurs at all in the rhizosphere of wheat growing on otherwise *Azotobacter*-free soil, it must be quite sporadically.

Other counts of *Azotobacter* were carried out in the rhizosphere of wheat growing on a soil favourable for growth of *Azotobacter* (No. 44, Table 2). A plot of about 25 square feet, upon which wheat was grown for demonstration purposes, was selected. Samples were taken from the beginning of September to the beginning of December, 1937, i.e. from the heading to the ripening stage of the wheat, with an additional sample in January, 1938, when the plot was left under stubble. Owing to the layout of the plots, samples could not be taken at a distance of more than 6 inches from the plants, so that the data refer only to "soil adjacent to roots" and "roots with adhering soil", the latter representing the rhizosphere (Thom and Humfield, 1932). At each sampling one or two plants were dug up to a depth of about 5 inches by cutting out a block of soil around their roots. The soil and roots were immediately brought to the laboratory and separated as completely as possible by shaking of the roots; platings were then carried out on the soil as well as the root material, of which another portion was used for moisture determination. Besides counts of *Azotobacter* on dextrine agar, some counts of other microorganisms were also made on glucose-casein agar (glucose 2·0 gm.; casein dissolved in n/10 NaOH, 0·2 gm.; K_2HPO_4 0·5 gm.; $MgSO_4$ 0·2 gm.; $FeCl_3$ 0·01 gm.; agar 20·0 gm.; H_2O 1000 c.c.; pH 6·5–6·6). Colonies of bacteria and actinomycetes were counted on this medium after incubation for 8 days at 28–30°C.

The results are found in Table 3. The numbers of *Azotobacter* in the soil itself during the vegetation period are much higher than in any of the soils from the wheat area, on 11th November even reaching 10,000 per gm.; in the rhizosphere the numbers are generally somewhat (up to 3 times) higher, but this is not a rule without exception. The counts on glucose agar show that the *Azotobacter*-population accounts for only an insignificant fraction of the total numbers of living microorganisms, not even including fungi and those bacteria that do not develop on the agar medium. This is especially the case in the rhizosphere, where the bacterial numbers are generally 5 to 10 times as high as in the soil, while the numbers of *Azotobacter* are but slightly increased; it is also of interest to note that the total numbers of bacteria in the rhizosphere are of an order of

TABLE 3

Azotobacter *in Soil and Rhizosphere of Wheat Plants.*

Date of Sampling.	H_2O % in Soil.	*Azotobacter* per gm. Dry Matter.		Ratio R/S.	Other Microorganisms. Mill. per gm.			
					Soil.		Rhizosphere.	
		Soil.	Rhizosphere.		Bact.	Act.	Bact.	Act.
9/4/37 (fallow)	10·5	1,640	—	—	—	—	—	—
6/9/37	11·2	3,700	10,800	2·7	—	—	—	—
13/9/37	8·2	1,610	4,050	2·5	—	—	—	—
20/9/37	7·3	4,100	1,270	0·31	—	—	—	—
28/9/37	5·1	1,920	3,310	1·7	84	10	586	14
5/10,37 ..	10·2	1,270	1,570	1·2	143	7	1,360	14
12/10/37 ..	10·4	2,160	1,870	0·87	107	13	740	30
20/10/37 ..	9·0	2,620	8,350	3·2	79	20	934	34
28/10/37 ..	15·2	1,790	4,300	2·4	138	8	940	27
10/11/37 ..	16·7	10,500	3,190	0·30	142	10	906	15
18/11/37 ..	9·6	1,460	2,860	2·0	98	8	(lost)	
26/11/37 ..	7·5	2,170	1,820	0·84	108	9	497	27
8/12/37 ..	8·8	1,510	2,020	1·3	83	10	494	33
18/1/38 (stubble)	9·5	550	—	—	29	10	—	—

magnitude comparable with those found by Starkey (1929), Gräf (1930), and Thom and Humfield (1932). These phenomena indicate clearly that the organic root secretions and sloughed-off root tissues are chiefly utilized by microorganisms other than *Azotobacter* (even under soil conditions favourable for this organism), and thus hardly serve as energy material for nitrogen fixation to any significant extent—in agreement with the findings of Krasilnikov (1934). We shall revert to this subject later.

(c). *Nitrogen-fixing Capacity of* Azotobacter *Isolated from Australian Soils.*

Azotobacter is relatively sparsely represented in Australian wheat soils. Before proceeding to the problem of their function in the soil itself, the actual nitrogen-fixing capacity of these organisms in pure culture may be discussed. This question has been widely studied in the past.

Contrary to the view commonly expressed in most earlier investigations, viz., that the fixation of elementary nitrogen is a process that involves the consumption of large quantities of free energy which has to be derived from the oxidation of carbon compounds, Burk (1934; see also Meyerhof and Burk, 1928) has shown by thermodynamical calculations that the fixation process as such requires little energy, and if this were the only physiological function of *Azotobacter*, the oxidation of 1·5 gm. of glucose to carbon dioxide and water would suffice for the fixation of 1 gm. of nitrogen. Bach et al. (1934) claim to have observed enzymic fixation of up to 0·279 gm. nitrogen per gm. of glucose—a yield which begins to approach the theoretical limit calculated by Burk. These findings, however, have not been confirmed by Roberg (1936) and Lineweaver (1938), and the final products of fixation are exclusively, or nearly so, represented by bacterial protoplasm. Since the cells of *Azotobacter* contain at least 4 to 5 times as much carbon as nitrogen, the theoretical yield is reduced to 80–100 mgm. nitrogen per gm. glucose, and even this limit cannot actually be attained, because a considerable proportion of the carbonaceous food is used for cell respiration

and given off as carbon dioxide, and there is often, besides the synthesis of nitrogenous cell substance, a formation of considerable amounts of non-nitrogenous intercellular substance (polysaccharides). Consequently, the yield of fixed nitrogen is in pure cultures under favourable conditions usually reduced to some 10 to 15 mgm. per gm. of glucose or mannite consumed. Higher yields have occasionally been reported. Certain strains, according to Hunter (1923), Kostytchev (1924), and Krishna (1928*a*) may fix 18–23 mgm. nitrogen per gm. glucose. (If we assume with Burk and Meyerhof (1928) and Burk and Lineweaver (1930) that the cell dry matter contains 10% N and 50% C, this would correspond to a utilization of about 23–29% of the glucose-carbon for cell synthesis, and the rest for other physiological activities, largely respiration; Ranganathan and Norris (1927) and Krishna (1928*a*) found that 65–72% of the glucose-carbon consumed was liberated as carbon dioxide). Krainsky (1910) grew *Azotobacter* in mannite solution on sand, and reported a remarkably efficient nitrogen fixation at low moisture content—1 mgm. nitrogen per 11 mgm. carbon liberated as CO_2, corresponding to fixation of about 36 mgm. nitrogen per gm. mannite used for respiration. Unfortunately no parallel experiments were made, and only the mannite-carbon liberated as carbon dioxide was taken into account (actually there was 0·5 gm. mannite present in the medium in which 4·12 mgm. nitrogen were fixed, and residual mannite was not determined at the end of the experiment); Krainsky's method of calculation thus makes the yield appear unduly high, and the experiment cannot be accepted as valid proof of a particularly efficient nitrogen fixation in soil of low moisture content (cf. also Traaen, 1916). Koch and Seydel (1912), in a frequently quoted contribution, found a surprisingly economic utilization of the energy material in young agar cultures of *Azotobacter*, where after 2 to 3 days up to 70–80 mgm. nitrogen were fixed per gm. of glucose consumed; in older cultures the sugar consumption continued without being accompanied by a corresponding nitrogen fixation. The maximal gain is surprising, since it implies that the cells must have grown practically without respiring (actually, young cultures of *Azotobacter* respire most intensely (Meyerhof and Burk, 1928)), or else there must have been an accumulation of fixation-products of very much higher nitrogen content than the cell material. Later investigations by Hunter (1923), Cutler and Bal (1926), Ranganathan and Norris (1927) and Bortels (1936) have affirmed the general principle of decreasing efficiency in older cultures, but have shown far less extraordinary gains in young cultures (16–20 mgm. N per gm. of glucose).

In sharp contrast to most workers, who state that good aeration of the cultures makes for increased efficiency of the fixation process (Koch and Seydel, 1912; Hunter, 1923), Meyerhof and Burk (1928) found the ratio (c.c. N_2 fixed/c.c. O_2 consumed) *increasing* with decreasing oxygen tension and corresponding increase in the partial pressure of nitrogen; they concluded that an economical fixation of nitrogen might be expected in deeper soil layers with an atmosphere of low oxygen content.

It has frequently been claimed that low concentrations of sugar are utilized more economically by *Azotobacter* than higher ones (Waksman, 1932). Observations by Hunter (1923) and Krishna (1928*a*), however, have not confirmed this. Finally it may be mentioned that molybdenum deficiency reduces not only the rate of fixation, but also the yield per unit of sugar (Bortels, 1936).

To test the general efficiency of the forms of *Azotobacter* that commonly occur in Australian soils, 24 strains were isolated, most from the plate counts

and solution tests in Table 2, and some from incubation experiments with soils with various additions of organic materials. When plate counts were made from these experiments, a number of organisms other than *Azotobacter* were sometimes seen to produce quite a fair growth on the dextrine agar, particularly in high dilutions where the colonies were well separated. Some of these organisms (20 bacteria, 5 actinomycetes, 1 yeast, and 11 filamentous fungi) were isolated and tested qualitatively for growth in a nitrogen-free glucose solution where *Azotobacter* grew well. None of the bacteria produced more than a barely visible trace of growth after incubation for 5 to 9 weeks at 28–30°C., whereas the actinomycetes and particularly some of the fungi grew comparatively well. Some of those organisms that seemed to grow best were tested quantitatively together with the 24 pure cultures of *Azotobacter*; the experiments also included tests with an impure, but *Azotobacter*-free culture of blue-green algae, and an *Azotobacter*-free mixture of bacteria from the rhizosphere of wheat (from plate count 26/11/1937; inoculum was obtained by scraping off the thin bacterial growth between the *Azotobacter*-colonies). The organisms were grown in the following solution (modified from Bortels, 1936): Glucose 20·0 gm.; K_2HPO_4 1·0 gm.; $MgSO_4$ 0·5 gm.; $FeCl_3$ 0·1 gm.; Na_2MoO_4 0·05 gm.; $CaCO_3$ 5·0 gm.; H_2O 1000 c.c.

The medium was used in portions of 25 c.c. (= 0·5 gm. glucose) in 100 c.c. round flat-bottomed flasks, where it formed a layer about 12 mm. deep. The flasks were sterilized at 10 lb. pressure for 15 minutes and inoculated from young (2–3 days) slope cultures on dextrine agar. The organisms other than *Azotobacter* were tested after only a few transfers since isolation, and had never been grown on any nitrogenous medium in order not to risk a loss of what nitrogen-fixing power they might possess (cf. Selim, 1931). Four series of experiments were run; in the last two the medium was given an extra addition of 0·1% agar. As shown by Rippel (1937), this accelerates the growth which, particularly in the second series, was often rather slow (influence of a "weather-factor" as suggested by Stapp and Bortels (1936)?). Incubation took place at 28–30°C., usually 2 or 3 weeks for *Azotobacter*, and 3 to 5 weeks for the other organisms; very rapidly growing *Azotobacter*-cultures were analysed after 6–8 days, when the *chroococcum*-strains showed the beginning of black pigmentation around the edges of the surface of the solution. Before analysis, the cultures were diluted to 50 c.c. after careful distribution of the growth by shaking with glass beads, 2 c.c. were withdrawn and tested qualitatively for residual glucose with Fehling's solution, and the rest was analysed for total nitrogen. Control flasks were analysed immediately after inoculation, as well as after incubation, in which latter case they were inoculated immediately before analysis (the inoculum, however, was in all cases so small that a micro-method would have been necessary to detect its influence on the nitrogen content of the medium).

The results of these experiments are seen in Table 4. Not only did the control flasks absorb no nitrogen compounds from the atmosphere,[14] but even some of the small quantity of nitrogen present as impurities (chiefly of the calcium carbonate in series 1 to 3) seems actually to be lost during the incubation (cf. M. Löhnis, 1930). Therefore the *initial* nitrogen content of the controls has been subtracted from that of the cultures as correction for nitrogen in medium plus inoculum. With one or two exceptions in the first series, the *Azotobacter*-strains all grew well,

[14] The same was the case with control solutions of H_2SO_4 and NaOH placed in the incubator.

TABLE 4.

Nitrogen-fixing Capacity of Azotobacter *and Other Soil Microorganisms.*

Culture.	Incubation Days.	Total N in Culture. Mgm.	Gain of N. Mgm.	Glucose in Medium After Incubation.	N Fixed. Mgm. per gm. Glucose Consumed.	Remarks.
Ser. 1. Jan.–Feb., 1937.						
Control unincubated ..	0	0·20				
Control incubated (*a*)	15	0·14				
(*b*)	32	0·10				
(*c*)	32	0·02				
Az. chroococcum 8 (*a*)	14	6·56	6·36	Neg.	*12·7*	
(*b*)	14	6·23	6·03	Neg.	*12·1*	
25 (*a*)	14	7·63	7·43	Neg.	*14·9*	
(*b*)	14	7·51	7·31	Neg.	*14·6*	
10 (*a*)	14	7·34	7·14	Neg.	*14·3*	
(*b*)	14	6·42	6·22	Neg.	*12·4*	
11 (*a*)	14	7·26	7·06	Neg.	*14·1*	
(*b*)	14	6·27	6·07	Neg.	*12·1*	
12 (*a*)	14	4·43	4·23	Faint.	*>8·4*	
(*b*)	14	5·37	5·17	Pos.	*>10·3*	
16 (*a*)	14	1·88	1·68	Pos.	*>3·3*	(*a*): Scant growth.
(*b*)	14	9·33	9·13	Neg.	*18·3*	
30 (*a*)	21	1·14	0·94	Pos.	*>1·9*	Very scant growth.
(*b*)	21	1·21	1·01	Pos.	*>2·0*	
Fusarium sp. 14 ..	32	0·25	(0·05)		(Nil)	Scant growth.
Bacterium sp. 11 (*a*) ..	21	0·11	(Nil)			Growth hardly visible.
(*b*) ..	21	0·14	(Nil)			
Ser. 2. May–June, 1937.						
Control unincubated (*a*)	0	0·15				
(*b*)	0	0·17				
Control incubated (*a*)	21	0·07				
(*b*)	21	0·04				
Az. chroococcum sand-kaolin mixture plus straw (Table 15) (*a*)	14	4·29	4·13	Faint.	*>8·3*	
(*b*)	14	4·83	4·67	(Trace)	*>9·3*	
Az. chroococcum 34 (*a*)	14	4·55	4·39	Pos.	*>8·8*	
(*b*)	14	7·51	7·35	Neg.	*14·7*	
43 (*a*)	14	7·21	7·05	Neg.	*14·1*	
(*b*)	14	6·62	6·46	Neg.	*12·9*	

* 5 c.c. conc. acid added immediately after inoculation.

Az. chroococcum 44 (*a*)	14	7·06	6·90	Neg.	*13·8*	
(*b*)	14	5·71	5·55	Neg.	*11·1*	
31 (*a*)	21	4·95	4·79	Neg.	*9·6*	Slow growth.
(*b*)	21	5·06	4·90	Neg.	*9·8*	
33 (*a*)	21	5·43	5·27	Neg.	*10·5*	Slow growth.
(*b*)	21	5·42	5·26	Neg.	*10·5*	
32 (*a*)	21	5·62	5·46	Neg.	*10·9*	Slow growth.
(*b*)	21	5·13	4·97	Neg.	*9·9*	
Az. beijerinckii(?), sand-kaolin mixture plus straw (*a*)	21	5·42	5·26	Neg.	*10·5*	Slow growth.
(*b*)	21	4·72	4·56	Neg.	*9·1*	
Bacillus sp., sand-kaolin mixture plus straw (*a*)	30	0·11	(Nil)			No visible
(*b*)	30	0·09	(Nil)			growth.
Actinomyces sp., sand-kaolin mixture plus straw (*a*)	21	0·06	(Nil)			Scant granular
(*b*)	21	0·09	(Nil)			growth.
Dematium pullulans (?) 23 (*a*)	21	0·10	(Nil)			Very scant
(*b*)	21	0·08	(Nil)			growth.
Dark green unidentified fungus 30 (*Helminthosporium* ?) (*a*) ..	24	0·13	(Nil)			Scant flaky
(*b*) ..	24	0·08	(Nil)			mycelium.
Crude culture of blue-green algae (cf. Table 27) (*a*)	28	0·10	(Nil)			Trace of
(*b*)	28	0·06	(Nil)			growth only.

Ser. 3. Nov.–Dec., 1937. 0·1% agar added to glucose solution.

Control unincubated (*a*)	0	0·17				
(*b*)	0	0·11				
Control incubated (*a*)	21	0·12				
(*b*)	21	0·11				
Control incubated + H_2SO_4*	20	0·06				
Az. chroococcum 49 (*a*)	8	5·69	5·55	Neg.	*11·1*	Very rapid
(*b*)	8	6·31	6·17	Neg.	*12·3*	growth.
48 (*a*)	12	6·07	5·93	Neg.	*11·9*	
(*b*)	12	5·89	5·75	Neg.	*11·5*	
66 (*a*)	18	4·84	4·70	(Trace)	*>9·4*	(*a*) : slow
(*b*)	14	7·37	7·23	Neg.	*14·5*	growth.
63 (*a*)	18	5·88	5·74	Neg.	*11·5*	
(*b*)	18	6·44	6·30	Neg.	*12·6*	
30 (*a*)	12	5·49	5·35	Neg.	*10·7*	Normal
(cf. Ser. 1). (*b*)	12	5·46	5·32	Neg.	*10·6*	growth.

TABLE 4.—*Continued.*

Culture.	Incubation Days.	Total N in Culture. Mgm.	Gain of N. Mgm.	Glucose in Medium After Incubation.	N Fixed. Mgm. per gm. Glucose Consumed.	Remarks.
Az. vinelandii	8	8·50	8·36	Neg.	*16·7*	Rapid growth.
Az.-free bacterial mixture from rhizosphere of wheat (*a*) ..	21	0·26	(0·12)	Pos.	?	Slight gas formation.
(*b*) ..	21	0·34	(0·20)	Pos.	?	
Ser. 4. July, 1938. 0·1% agar added to glucose solution.						
Control unincubated (*a*)	0	0·08				
(*b*)	0	0·06				
Control incubated ..	14	0·05				
Az. vinelandii (*a*) ..	6	6·92	6·85	Neg.	*13·7*	Very rapid growth.
(*b*) ..	6	6·43	6·36	Neg.	*12·7*	
Az. chroococcum 79 (*a*)	6	5·60	5·53	Neg.	*11·1*	Very rapid growth.
(*b*)	6	6·42	6·35	Neg.	*12·7*	
80 (*a*)	12	6·01	5·94	Neg.	*11·9*	
(*b*)	12	6·06	5·99	Neg.	*12·0*	
81 (*a*)	14	5·22	5·15	Neg.	*10·3*	
(*b*)	14	4·98	4·91	Neg.	*9·8*	
Az. chroococcum from mixed soil No. 31+33 + wheat straw (Table 19) (*a*) ..	12	6·11	6·04	Neg.	*12·1*	
(*b*) ..	12	6·05	5·98	Neg.	*12·0*	
Yellow mucoid bacterium from synthetic soil+water-extracted wheatstraw (Table22)	21	0·01	(Nil)			Trace of growth only.
White bacterium from synthetic soil+untreated wheat straw (Table 22)	21	0·02	(Nil)			Growth not visible.

in most cases consuming all the glucose within 2–3 weeks and fixing 9 to 18 mgm. N per gm. glucose consumed. Where some glucose is left, the amount of fixed nitrogen generally corresponds to about 8 mgm. per gm. of glucose supplied. Duplicate cultures show a good agreement, with only few exceptions (strain 16, ser. 1; strain 34, ser. 2; strain 66, ser. 3). Strain No. 30, which almost failed to grow in the first series, showed normal growth and fixation on re-trial in solution with agar; it may be that this strain is particularly sensitive either to the accelerating influence of colloids (Rippel, 1937) or to a possible weather-factor (Stapp and Bortels, 1936).

The other organisms failed entirely to fix nitrogen under the conditions of the experiment, with the possible exception of the bacterial mixture from the wheat rhizosphere; there is here a mere trace of nitrogen fixation, accompanied by a slight gas formation; possibly some spores of clostridia had been lying dormant on the plates and had become introduced with the inoculum. The fungi, and particularly the one from soil No. 30 (a *Helminthosporium*?), produced a quite appreciable growth, but this is obviously due merely to ability to utilize the nitrogenous impurities of the medium, and not to nitrogen-fixing capacity.

An additional set of experiments was carried out to test whether the present *Azotobacter*-strains would fix nitrogen with particular efficiency in quite young cultures. In order to make conditions as favourable as possible, a very dilute glucose solution was used. In the first series it contained half the concentration of soluble salts of that of the previous experiments, in addition to 0·125% glucose, 0·1% agar, and 0·1% $CaCO_3$; portions of 80 c.c. (= 0·1 gm. glucose) were placed in wide flat-bottomed flasks, where the liquid formed a layer about 5 mm. deep. In the second series the basal solution was of the normal composition, with 0·2% glucose; 75 c.c. portions were used in round flasks of 1 litre capacity. Two normally growing strains of *Az. chroococcum* were tested in the first series, *Az. vinelandii* and a rapidly growing *Az. chroococcum* in the second. Controls were analysed immediately after inoculation only; in the first series an extra flask with sugar-free medium was included in order to see if the agar contained any available energy material; this was not found to be the case. After incubation for 48–96 hours at 28–30°C., the cultures were diluted to 100 c.c., of which 10 c.c. were tested for glucose and the rest analysed for nitrogen. The results are found in Table 5.

TABLE 5.

Nitrogen Fixation by Azotobacter *in Solution with Low Concentration of Glucose.*

Culture.	Incubation Hours.	Total N in Culture. Mgm.	Gain of N. Mgm.	Glucose in Medium After Incubation.	N Fixed. Mgm. per gm. Glucose Consumed.
Ser. 1. July, 1937. 80 c.c. 0·125% glucose-solution in flat-bottomed flasks.					
Control solution (*a*) ..	0	0·07			
(*b*) ..	0	0·09			
Az. chroococcum 44 (*a*) ..	48	1·22	1·14	Neg.	*11·4*
(*b*) ..	48	1·17	1·09	Neg.	*10·9*
34 (*a*) ..	48	0·87	0·79	Neg.	*7·9*
(*b*) ..	48	0·95	0·87	Neg.	*8·7*
Az. chroococcum— glucose-free solution ..	48	0·10	(Nil)		
Ser. 2. July, 1938. 75 c.c. 0·2% glucose solution in large round flasks.					
Control solution (*a*) ..	0	0·07			
(*b*) ..	0	0·09			
(*a*)	48	1·55	1·47	(Trace)	(*10·3*)
Az. vinelandii (*b*) ..	72	1·74	1·66	Neg.	*11·1*
(*c*)	96	1·76	1·68	Neg.	*11·2*
Az. chroococcum 79 (*a*) ..	48	1·47	1·39	Neg.	*9·3*
(*b*) ..	72	1·68	1·60	Neg.	*10·7*

There is absolutely no indication of any increased efficiency of nitrogen fixation in these experiments, although the conditions should here be in every respect optimal: short incubation time and low concentration of glucose to avoid accumulation of metabolic products in the medium, presence of a colloid, and a fully sufficient concentration of molybdenum (Bortels, 1936). In fact, strains 34 in the first series and *Az. vinelandii* in the second seem to fix less nitrogen per gm. glucose here than in the 2% solution with longer incubation. Koch and Seydel's (1912) observations are thus certainly not generally valid. There is a good reason to suspect that the lower efficiency in older cultures, as well as the less economical utilization of higher concentrations of sugar or mannite sometimes reported, may really have been caused by molybdenum deficiency of the medium; it is noteworthy that all these findings have been reported prior to the discovery of the function of molybdenum in nitrogen fixation by Bortels in 1930.

As a whole the experiments show that the *Azotobacter* generally occurring in Australian soils have a perfectly normal nitrogen-fixing capacity, and do not differ greatly from each other. The occasionally appearing *Az. beijerinckii* and *vinelandii* do not differ much in this respect from the common *Az. chroococcum*. The importance of these organisms in the nitrogen economy of the soil will thus presumably depend less upon strain-specificity than upon the quantity of energy material which they actually consume.

(*d*). *Nitrogen Fixation in Soils under Laboratory Conditions.*

The foregoing results, viz., that *Azotobacter* is usually either absent or only sparsely represented in the wheat soils, that the commonly occurring strains have no extraordinary nitrogen-fixing power, and that no other aerobic nitrogen-fixing microorganisms appear to be present, might seem to speak strongly against non-symbiotic nitrogen fixation as a factor of significance. Before drawing any final conclusions, however, we must turn to the method of nitrogen fixation experiments in the soil itself. Firstly, colony counts of *Azotobacter* may be counts of cell aggregates rather than of individual cells, and we know little about the relation between these counts and the actual fixation of nitrogen. Further, organisms of the butyric acid bacilli group may be capable of acting in the soil even at moderate degrees of moisture (Winogradsky, 1925), and finally we must not overlook the possible existence of organisms capable of fixing nitrogen when growing in the soil but not in the ordinary artificial media. In soils incubated under favourable conditions of moisture and temperature and with addition of such organic materials as might come into consideration as energy materials under natural conditions, all such organisms, both known and unknown, should be expected to find chances for displaying their activities. Several series of experiments have been undertaken from this point of view.

1. *Soils without Addition of Organic Matter.*

In looking for organic compounds that might serve as food substance for nitrogen-fixing bacteria, one would naturally first think of the organic matter normally present in the soil, either as still incompletely decomposed plant residues or as structureless "humus". Numerous attempts have been made to prove that a measurable fixation of nitrogen may take place on the basis of these materials. The first of these were the fundamental researches by Berthelot (1888–90), who in his first investigations on soils very poor in organic matter found increases of up to 40% of the nitrogen content after several months, but

later with more normal soils found more moderate gains of 5–15%. Nitrogen determinations were usually carried out only in duplicate by means of the now abandoned soda-lime method, occasionally controlled by the Dumas method. Most experiments were made on soil exposed to daylight, under laboratory, greenhouse or field conditions. Practically all subsequent investigators have used the Kjeldahl method in its various modifications for the nitrogen determinations. The following authors have reported gains of nitrogen in soils incubated under laboratory conditions without addition of organic matter:

Schneider (1906), in Germany, incubated soil from 15 differently fertilized plots for 6 weeks at room temperature and found gains of nitrogen in all cases—from 52 to 251 p.p.m., or 6 to 33% of the original nitrogen content. The analytical method was not described in detail.

Warmbold (1906), in Germany, reported small but irregular gains of nitrogen under similar conditions, even in sterilized soil. These statements were vigorously contested by Pfeiffer et al. (1906), and in later experiments Warmbold (1908) found definite losses of nitrogen by incubation of sterilized soil.

Koch et al. (1907), in Germany, found no significant increase in nitrogen content of soil incubated for up to 4 months at room temperature, but sometimes large gains (120–130 p.p.m.) after 8–10 months; the analytical error was clearly stated. The authors regard it as uncertain whether the effect was due to absorption of ammonia from the atmosphere or to other causes.

Krainsky (1908), in Russia, reported fixation of up to 130 p.p.m. nitrogen in soil kept for 3 months at room temperature. The gain was highest at low moisture content, where it was calculated that one part of nitrogen had been fixed for every 9 parts of carbon liberated as CO_2. Only two parallel determinations of nitrogen were made, and the method was not described in detail.

Remy (1909), in Germany, found gains of nitrogen, clearly exceeding the analytical error, in soils incubated 25 to 70 days at room temperature. The largest absolute gain (91·5 p.p.m., or about 10% of the original nitrogen content) was found in loam with addition of $CaCO_3$, but the largest relative gain (38·4 p.p.m., or 25% of the original) in $CaCO_3$-treated sand soil poor in humus and apparently devoid of *Azotobacter*. The gains were sometimes followed by losses. Some factor other than biological fixation seems to have been operating, as certain gains of nitrogen were sometimes observed where mercury chloride had been added to the soil (cf. de' Rossi, 1932*c*).

Lemmermann and Wichers (1914), in Germany, observed a gain of nitrogen (63·4 p.p.m., or about 16% of the original content) in a soil which served as control in a denitrification experiment. In several other series no significant changes were observed. The authors do not discuss these findings further.

Mockeridge (1917), in England, found gains of 110 to 200 p.p.m. nitrogen in two soils incubated for two weeks at room temperature. The soils were rich in nitrogen, and the gains represent only 4 to 6% of the original content. The differences appear significant, but the procedure of analysis was not described in detail. With addition of "bacterised peat" even larger gains were reported.

Zoond (1926), in England, reported similar gains (300 p.p.m., or about 8%) in a humus-rich soil incubated for 4 weeks at 25°C. with addition of 1% $CaCO_3$.

de' Rossi (1932*c*), in Italy, mentioned briefly an experiment suggesting nitrogen fixation by physico-chemical agencies in soil after heating or with addition of $HgCl_2$. Very small samples of soil (10 gm.) were used, and it is not clearly stated whether they were all analysed in a dry or moist condition.

Fehér (1933), in Hungary, worked on an initially strongly acid soil, which was sterilized, re-infected, and incubated for 13 months at different temperature, with and without restoration of the moisture content. At 15°C. large gains of nitrogen were found: in moist soil 60 p.p.m., or 13%, and in dry soil 106 p.p.m., or 24% of the initial content. At higher temperatures there was a tendency to loss of nitrogen (very marked at 55°C.). The method and error of analysis were not mentioned.

Sackett (1912) and Headden (1911–21), in Colorado, observed large gains of nitrogen in soils kept for 4 to 7 weeks at 28–30°C. Sackett found the strongest fixation at low degrees of moisture. Headden reported gains simultaneously with strong nitrate production, and stated that the gains could rise to 610 p.p.m., or nearly 50% of the initial content. Later, Headden (1922) observed gains of nitrogen in almost pure sand, corresponding to 10 times the initial content. He ascribed both the nitrogen fixation and the nitrification to the activity of *Azotobacter*. None of the authors describe the procedure of analysis in detail.

Greaves (1914), in Utah, incubated 16 soils for 6 weeks at 30°C., and reported gains of up to 168 p.p.m. nitrogen. In another contribution Greaves (1916) reported gains of 49 to 168 p.p.m. nitrogen in 3 soils incubated for 18 days at 30°C. One of these soils had continued to give good yields of wheat without nitrogenous fertilizers for 23 years, although it was poor in humus. Later again, Greaves and Nelson (1923) reported gains of 36 to 55 p.p.m. nitrogen in a calcareous soil incubated for 3 to 6 weeks at 28°C.; longer periods of incubation gave irregular results. (In all these experiments it appears that the gain of nitrogen was calculated as difference between nitrogen contents of untreated and sterile—presumably autoclaved—soil incubated under the same conditions.) In experiments with larger quantities of the same soil kept for 437 days at room temperature, Greaves and Nelson also found large but rather irregular gains, after 244 days even 160 p.p.m., or nearly 11% of the initial nitrogen content. The authors comment that "this soil in little less than one year had gained at the rate of 480 pounds of nitrogen per acre-foot of soil". The analytical error was not stated.

Fulmer (1917), in Wisconsin, gave figures suggesting a gain of 100 to 170 p.p.m. of nitrogen, or 4 to 6% of the initial, in field soil incubated for 6 weeks at 25°C., but stated cautiously that this might be due to the error of analysis.

Neller (1920), in New Jersey, mentioned briefly a gain of 7·7 to 9·4 mgm. nitrogen (in 200 gm. of soil?) in 4 soils from a fertilizing experiment. No further comments were made.

Murray (1921), in Washington, carried out nitrogen fixation experiments with straw; in the control soils he reported gains corresponding to about 17% of the original nitrogen content, which gains, in another experiment with addition of ammonium sulphate, even rose to 40%. Apparently only two parallel determinations were made, and the procedure of analysis was not described.

Lipman and Teakle (1925), in California, reported gains corresponding to 10–12% of the initial nitrogen content in both sterilized and unsterilized soil inoculated with *Azotobacter*. The differences are clearly significant, but it is not definitely stated whether sterilized or untreated soil served as control, nor was the analytical method clearly described.

Vandecaveye and Villanueva (1934) and Vandecaveye and Allen (1935), in Washington, incubated wheat soils for 115 to 165 days at room temperature and found large gains of nitrogen, sometimes exceeding 10% of the initial content

and accompanied by very small losses of carbon. Counts of *Azotobacter* and determinations of nitrate were also carried out. The authors remark (1935) that the gains could not be attributed to *Azotobacter*, which only occurred in comparatively low numbers. The analytical error was not stated.

Turk (1936), in Michigan, found gains of 80 to 222 p.p.m. nitrogen in 4 out of 6 wheat soils incubated for 12 weeks. One of the soils had pH 5·0 and contained no *Azotobacter*. The actual nitrogen contents of the soils, as well as the method of analysis, were not stated.

Walton (1915), in Punjab, reported a gain of about 6% of the original nitrogen content of a soil incubated for 3 weeks at 30°C. No further details were given.

Wilsdon and Ali (1922), in Punjab, found gains of up to 45% of the initial nitrogen content of soils in a series of experiments under conditions that were not closely defined. They concluded that the season was a factor of prime importance, and that soils tended to fix nitrogen, especially after long periods of drought.

Lander and Ali (1925) followed up these experiments and determined nitrogen in soils kept for up to 5 months at 30 to 35°C. In some cases they observed large gains of nitrogen, even increases from 0·0470 to 0·0823% N within one month; in other cases similar losses were found. These phenomena were interpreted as alternating nitrogen fixation and denitrification, although no bacteriological investigations were made. In spite of the irregularity of the results, the authors conclude that "Nature has provided a certain degree of remedy for the losses of nitrogen which take place during the removal of crops". The analytical method was not described in detail.

Sahasrabuddhe and Daji (1925) and Sahasrabuddhe and Ghatikar (1931), in Bombay, found similar results with soils incubated 2 or 4 weeks at 20 to 40°C.; gains of nitrogen were even observed in soil that had been heated to 100°C. (cf. de' Rossi, 1932*c*) and in soil containing as much as 62 p.p.m. of NO_3–N. Here, again, the method of analysis was not described in detail. Sahasrabuddhe and co-workers, as well as the previous Indian investigators, stress the importance of root and stubble remains of the crops as energy material for nitrogen fixation, without, however, carrying out any biological experiments.

De and Pain (1936), in Bengal, incubated 6 rice soils for 2 months at 33°C.; 4 soils showed gains corresponding to 40 to 120 lb. nitrogen per acre (the actual analytical data were not given). The highest gain was reported in a soil of pH 5·1 and not containing *Azotobacter*, which in the other soils occurred in numbers up to 2700 per gm. The authors think that this indicates the existence of still unknown but highly efficient nitrogen-fixing soil microorganisms.

Records of negative findings under similar conditions are comparatively few, but upon the whole better documented. Schloesing *père* (1888), in France, found no significant increases in nitrogen content of various soils after storage for 1–2 years; these experiments are interesting because of the systematic use of direct gasometric methods. Thiele (1905), in Germany, found similar results in sterilized soil inoculated with *Azotobacter* and incubated at 30°C., as well as in unsterilized soil stored in flasks under field conditions. In numerous experiments where the method of analysis was described in detail and the analytical error definitely stated, Lemmermann and Blanck (1908), Lemmermann, Blanck and Staub (1910), Lemmermann and Themlitz (1934), in Germany, and Christensen (1927), in Denmark, found generally no significant gains of nitrogen

in soils incubated without addition of energy material; occasionally there was indeed also in these experiments a small but apparently significant increase in nitrogen content; none of the authors, however, have explicitly ascribed this phenomenon to nitrogen fixation. (In the analytical data of Lemmermann et al. (1910) one notices a remarkably high content of NO_3– and NH_4–N, which could hardly be conducive to fixation.) de' Rossi (1932*d*), in Italy, found no gain of nitrogen in two soils incubated without addition of mannite for 10–12 days at 32°C., in spite of an appreciable multiplication of *Azotobacter*. In India, Meggitt (1923) found no gain of nitrogen in a soil which fixed large amounts on addition of sugar, and Bal (1928) observed only small and occasional gains which he did not consider significant.

Even if the extraordinary gains reported by Indian investigators may be fictitious, as pointed out by Bal (1925–28), the bulk of the evidence still seems to suggest that an intensive nitrogen fixation may, especially in soils from hot and dry climates like India and the western United States, take place at the expense of the organic matter normally present in the soil. If this is the case, the comparatively small amounts of organic matter must obviously be utilized much more economically than in pure cultures, whether by *Azotobacter* or by other organisms possibly capable of nitrogen fixation. It will appear from the survey given above, that the reported gains of nitrogen have largely been of the order of 5 to 10% of the original nitrogen content, or even higher; this is well beyond what we may expect to be able to detect analytically, and, if really existing, it would fully meet the nitrogen requirements of the crops. Experiments were therefore carried out in order to ascertain whether such processes take place in Australian wheat soils.

Thirty-three of the soils in Table 2 were used for this purpose. Portions of 120 to 180 gm. of air-dried, finely ground and sieved soil were moistened with distilled water to approximately 60% of their water-holding capacity,[15] placed in big Petri dishes (13·5 × 2 cm.), and incubated at 28–30°C. for 30 days, during which time the moisture content was restored every 2 to 3 days by addition of distilled water. Before and after incubation total nitrogen was determined (as well as ammonia and nitrate, which will be discussed later); in most cases counts of *Azotobacter* were also made. The experiments were carried out in duplicate, except with soils No. 1 and 2, the experiments on which were of a more tentative character. Only one dish with soil No. 9 was analysed, since this soil was abnormally rich in humus and was only included for the sake of comparison. Two soils, Nos. 6 and 7, were also analysed after 60 days, the second analysis being carried out on a mixture of equal parts of soil from the two parallels in the first period of incubation. Soil No. 8, which was of alkaline reaction, poor in humus and containing *Azotobacter*, was used in other experiments with addition of organic matter as a control soil designed to show optimal conditions for nitrogen fixation; it was therefore given an addition of 0·2% $CaHPO_4$. Soils No. 14 and 15 were exceptionally rich in nitrate at the outset of the experiment, which was therefore repeated: after incubation for 30 days, the soil which had been extracted with water for determination of nitrate was air-dried, re-moistened, and incubated

[15] It is difficult to get reliable estimates of the water-holding capacity, which may serve for comparison of light sand soils and very heavy clay soils, both of which types were represented in this investigation. It was therefore deemed preferable to rely on a subjective estimation of about 60% saturation by adding the water very slowly in small portions, until the soil had attained a fine crumbly texture without any stickiness.

for another period of 30 days, equal parts of soil from the two parallel dishes in the first period being used. Soil No. 35 deserves particular mention. Unlike the other samples, it was not taken to the depth of 6 inches, but represents the soil adhering to the roots of dead wheat plants (left without stubble-burning from harvest 1936 until the end of March 1937) to a depth of 2–3 inches, and was deliberately taken in such a way as to include a large proportion of dead root-material; an extra experiment was carried out on this soil with addition of 0·5% $CaCO_3$ and 0·025% Na_2HPO_4; this, together with the presence of large amounts of partially undecomposed plant material, should give particularly favourable conditions for nitrogen fixation. Soil No. 44 is somewhat comparable with this; it was not the same sample as in Table 2, but represents the soil adhering to the roots of wheat plants in the heading stage (13/9/1937, Table 3), and contained a certain amount of fine rootlets.

TABLE 6.

Numbers of Azotobacter *in Soils During Incubation at 28–30° C.*

(s+) and (s−) denote presence and absence, respectively, of *Azotobacter* in mannite solution.

Soil No. and pH.	Incubation Days.	*Azotobacter* per gm.	Soil No. and pH.	Incubation Days.	*Azotobacter* per gm.
31 (pH 6·5)	0 30	0 (s+) 13 (s+)	35 (pH 6·0)	0 30	0 (s−) 0 (s+)
32 (pH 6·1)	0 30	0 (s−) 5 (s−)	(35+$CaCO_3$)	30	(*a*) 66,500 (*b*) 54,300
24 (pH 7·4)	0 30	(s+) 0	34 (pH 7·6)	0 30	7 (s+) 18 (s+)
8 (pH 7·4)	0 30	9 (*a*) 5,900 (*b*) 4,400	15 (pH 5·4)	0 30	0 (s−) 0 (s−)
19 (pH 6·0)	0 30	(s−) 3 (s−)	23 (pH 7·6)	0 30	0 (s+) 0 (s+)
12 (pH 5·7)	0 30	67 (s+) 0 (s−)	51+52 (pH 6·4)	0 30	0 (s−) 0
25 (pH 6·5)	0 30	0 (s+) 0 (s+)	29 (pH 7·6)	0 28	0 (s+) 0 (s+)
21 (pH 5·9)	0 7 30	0 (s−) 0 0 (s−)	30 (pH 7·7)	0 30	(s+) 0
11 (pH 6·5)	0 30	0 (s+) 220 (s+)	26 (pH 7·5)	0 30	7 (s+) 140 (s+)
10 (pH 6·4)	0 30	20 (s+) 8 (s+)	16 (pH 7·1)	0 30	0 (s+) 640 (s+)
13 (pH 6·1)	0 30	0 (s+) 0 (s+)	44 (pH 7·4)	0 15 30	2,040 (s+) 2,190 2,340
20 (pH 6·0)	0 30	0 (s−) 0 (s+)	9 (pH 6·9)	0 30	590 (s+) 210 (s+)
14 (pH 5·6)	0 30	3 (s−) 0 (s−)			

The results of the *Azotobacter*-counts are shown in Table 6. A really striking increase in *Azotobacter* has taken place only in soil No. 8, to which $CaHPO_4$ had been added, and in No. 35 with addition of lime and phosphate. Otherwise we find only small increases, as in Nos. 11, 16, and 26, or none that can be considered significant at all. In the cases where *Azotobacter* were originally present in soil of pH less than 6·0 (Nos. 12 and 14) they appear to have died out during incubation, as might be expected (cf. Christensen, 1915; Gainey, 1923; Vandecaveye and Anderson, 1934); special acid-resistant forms thus do not seem to occur in these soils. Upon the whole the results agree with those of other investigators (de' Rossi, 1932*d*; Vandecaveye et al., 1934–35) in showing that the numbers of *Azotobacter* do not generally rise to a much different order of magnitude during incubation under favourable conditions of moisture and temperature, even in soils of neutral to alkaline reaction.

Table 7 shows the results of the nitrogen determinations. The significance of the difference in nitrogen content of soil before and after incubation was tested by applying the *t*-test of Fisher (1930, p. 107) for the comparison of two means. For this test we calculate the following statistics:

(1) Mean of determinations of nitrogen,

$$\bar{x} = \frac{S(x)}{n+1},$$

where $n + 1$ represents the number of parallel determinations, and $S(x)$ the sum of individual determinations.

(2) Variance resulting from pooling the sums of squares of deviations from the two means,

$$s^2\left(\frac{1}{n_1+1} + \frac{1}{n_2+1}\right) = \frac{(n_1+n_2+2)\left(S(x_1-\bar{x}_1)^2 + S(x_2-\bar{x}_2)^2\right)}{(n_1+1)\ (n_2+1)\ (n_1+n_2)}$$

and hence the standard deviation

$$s = \sqrt{\frac{S(x_1-\bar{x}_1)^2 + S(x_2-\bar{x}_2)^2}{n_1+n_2}},$$

(3) and $t = \frac{\bar{x}_2-\bar{x}_1}{s}\sqrt{\frac{(n_1+1)\ (n_2+1)}{n_1+n_2+2}}$;

(4) $n = n_1 + n_2$,

where the indices *1* and *2* denote the two sets of estimations to be compared. From Fisher's Table iv the probability, P, of the difference being significant was found; if the value of P is less than 0·05 the difference may be regarded as significant. The data given in Table 7 are: the mean of the nitrogen determinations ($\bar{x}$), the number of parallel determinations ($n + 1$), sum of squares of deviations from the mean, ($S(x-\bar{x})^2$), apparent change in nitrogen content as difference between means of determinations before and after incubation ($\bar{x}_2-\bar{x}_1$), values of *t* calculated from these data, and the corresponding values of P. The pH values are also given, and in the final column the apparent change in nitrogen content as percentage of initial total nitrogen content ($100(\bar{x}_2-\bar{x}_1)/\bar{x}_1$), where $\bar{x}_1$ and $\bar{x}_2$ represent the means before and after incubation. In the table the soils are arranged in order of increasing nitrogen content.

TABLE 7.

Changes in Nitrogen Content of Soils Incubated 30 d. 28–30° C. without Addition of Organic Matter.

Soil No.	pH.	Total N, p.p.m., Mean. ($\bar{x}$).	n+1.	S $(x-\bar{x})^2$	Gain (+) or Loss (−) of N, p.p.m. ($\bar{x}_2-\bar{x}_1$).	t.	P.	Gain or Loss of N in % of Total Initial N.
31	6·5	*I 182·3	4	56·8				
		*F (*a*) 181·7	3	8·7	−0·6	0·287	0·8−0·7	−0·33
		F (*b*) 186·7	3	16·7	+4·4	1·987	0·2−0·1	+2·41
33	6·2	I 227·7	3	4·7				
		F (*a*) 228·0	3	8·0	+0·3	0·207	0·9−0·8	+0·13
		F (*b*) 227·5	4	121·0	−0·2	0·076	1·0−0·9	−0·09
32	6·1	I 243·0	4	82·0				
		F (*a*) 245·7	3	4·7	+2·7	1·012	0·4−0·3	+1·11
		F (*b*) 241·0	3	14·0	−2·0	0·814	0·5−0·4	−0·82
24	7·2	I 390·8	4	102·8				
		F (*a*) 395·0	3	54·0	+4·2	1·240	0·3−0·2	+1·07
		F (*b*) 393·0	3	122·0	+2·2	0·570	0·6−0·5	+0·56
8	7·4	I 451·0	4	150·0				
		F (*a*) 453·3	3	112·7	+2·3	0·549	0·7−0·6	+0·51
		F (*b*) 437·8	4	82·7	−13·2	2·176	0·1−0·05	−2·93
19	6·0	I 457·7	3	10·7				
		F (*a*) 450·3	3	153·7	−7·4	1·224	0·3−0·2	−1·62
		F (*b*) 457·7	3	44·7	0	—	—	0·00
12	5·7	I 531·0	3	74·0				
		F (*a*) 527·7	3	12·7	−3·3	0·872	0·5−0·4	−0·62
		F (*b*) 536·5	4	233·0	+5·5	0·919	0·4−0·3	+1·04
25	6·5	I 533·8	5	436·6				
		F (*a*) 529·0	3	104·0	−4·8	0·640	0·6−0·5	−0·90
		F (*b*) 545·3	3	8·7	+11·5	1·828	0·2−0·1	+2·15
21	5·9	I 582·0	3	32·0				
		F (*a*) 580·3	3	50·7	−1·7	0·269	0·9−0·8	−0·29
		F (*b*) 583·5	4	291·0	+1·5	0·244	0·9−0·8	+0·26
11	6·5	I 628·6	5	755·4				
		F (*a*) 633·3	4	708·8	+4·7	0·485	0·7−0·6	+0·75
		F (*b*) 640·4	5	859·2	+11·8	1·313	0·3−0·2	+1·87
10	6·4	I 632·8	4	486·8				
		F (*a*) 630·7	3	242·7	−2·1	0·228	0·9−0·8	−0·33
		F (*b*) 632·0	3	282·0	−0·8	0·085	1·0−0·9	−0·13
13	6·1	I 641·0	5	730·0				
		F (*a*) 642·3	3	12·7	+1·3	0·160	0·9−0·8	+0·20
		F (*b*) 646·3	3	4·7	+5·3	0·656	0·6−0·5	+0·83
20	6·0	I 655·8	4	523·6				
		F (*a*) 649·5	4	705·0	−6·3	0·623	0·6−0·5	−0·96
		F (*b*) 655·0	3	86·0	−0·8	0·095	1·0−0·9	−0·12

* I = initial. F = final (after incubation).

TABLE 7.—*Continued.*

Soil No.	pH.	Total N, p.p.m., Mean. ($\bar{x}$).	n+1.	S $(x-\bar{x})^2$	Gain (+) or Loss (−) of N, p.p.m. ($\bar{x}_2-\bar{x}_1$).	t.	P.	Gain or Loss of N in % of Total Initial N.
14	5·6	I 660·2	5	1040·6				
		F (*a*) 643·0	4	238·0	−17·2	1·897	0·1−0·05	−2·61
		F (*b*) 639·7	3	108·7	−20·5	2·028	0·1−0·05	−3·11
Do. washed	6·0	I 627·0	3	14·0				
		F 621·0	2	8·0	−6·0	2·427	0·1−0·05	−0·97
35	6·0	I 712·2	6	838·6				
		F (*a*) 717·3	3	25·7	+5·1	0·649	0·6−0·5	+0·72
		F (*b*) 716·7	3	85·7	+4·5	0·554	0·7−0·6	+0·63
Do. +$CaCO_3$	,,	I 711·9‡	(6)	(838·6)				
		F (*a*) 720·8	4	74·8	+8·9	1·290	0·3−0·2	+1·25
		F (*b*) 699·3	3	12·7	−12·6	1·654	0·2−0·1	−1·81
34	7·6	I 744·0	3	14·0				
		F (*a*) 741·7	3	52·7	−2·3	0·690	0·6−0·5	−0·31
		F (*b*) 749·0	3	54·0	+5·0	1·485	0·3−0·2	+0·67
7	5·1	I 745·3	3	12·7				
		F (*a*) 729·5	4	1937·2	−15·8	1·048	0·4−0·3	−2·12
		F (*b*) 724·0	4	1338·0	−21·3	1·658	0·2−0·1	−2·86
		(*a*)+(*b*):						
		60 d. 682·3	3	80·7	*−63·0*	15·97	*<0·01*	*−8·46*
15	5·4	I 753·3	3	140·7				
		F (*a*) 754·7	3	18·7	+1·4	0·272	0·8−0·7	+0·19
		F (*b*) 761·0	3	98·0	+7·7	1·221	0·3−0·2	+1·02
Do. washed.	5·9	I 709·7	3	182·7				
		F 709·0	3	26·0	−0·7	0·119	1·0−0·9	−0·10
23	7·6	I 754·0	3	62·0				
		F (*a*) 744·0	4	626·0	−10·0	1·116	0·4−0·3	−1·33
		F (*b*) 749·7	3	16·7	−4·3	1·203	0·3−0·2	−0·57
1	6·0	I 795·7	3	440·7				
		F 802·7	3	164·7	+7·0	0·697	0·6−0·5	+0·88
27	5·8	I 796·0	4	493·0				
		F (*a*) 785·0	3	42·0	−11·0	1·392	0·3−0·2	−1·38
		F (*b*) 790·0	3	18·0	−6·0	0·770	0·5−0·4	−0·75
22	5·8	I 815·7	3	52·7				
		F (*a*) 813·0	3	122·0	−2·7	0·199	0·9−0·8	−0·33
		F (*b*) 811·3	3	88·7	−4·4	0·071	0·4−0·3	−0·54
51+52†	6·4	I 816·0	4	170·0				
		F (*a*) 822·7	3	40·7	+6·7	1·355	0·3−0·2	+0·82
		F (*b*) 813·7	3	32·7	−2·3	0·473	0·7−0·6	−0·28

‡ Corrected for introduction of 6·8 p.p.m. N in the $CaCO_3$.

† Mixture of equal parts of samples.

6	5·6	I 825·5	4	657·0				
		F (*a*) 820·0	4	2040·0	−5·5	0·355	0·8−0·7	−0·67
		F (*b*) 835·3	3	10·7	+9·8	1·119	0·4−0·3	+1·19
		(*a*)+(*b*):						
		60 d. 806·0	3	416·0	−19·5	1·744	0·2−0·1	−2·36
29	7·6	I 969·3	4	224·8				
		F (*a*) 964·3	3	44·7	−5·0	0·892	0·5−0·4	−0·52
		F (*b*) 957·3	3	2·7	−12·0	2·384	0·1−0·05	−1·24
30	7·7	I 1071·7	3	48·7				
		F (*a*) 1074·0	3	104·0	+2·3	0·456	0·7−0·6	+0·22
		F (*b*) 1069·8	4	1160·6	−1·9	0·160	0·9−0·8	−0·18
2	6·5	I 1252·7	3	788·7				
		F 1261·0	3	1116·0	+8·3	0·466	0·7−0·6	+0·66
26	7·5	I 1272·3	3	308·7				
		F (*a*) 1258·0	3	234·0	−14·3	1·504	0·3−0·2	−1·12
		F (*b*) 1265·3	3	104·7	−7·0	0·843	0·5−0·4	−0·55
28	5·7	I 1582·0	4	534·0				
		F (*a*) 1578·0	3	378·0	−4·0	0·388	0·8−0·7	−0·25
		F (*b*) 1566·0	3	194·0	−16·0	1·777	0·2−0·1	−1·01
16	7·1	I 1594·5	4	211·0				
		F (*a*) 1599·7	3	160·7	+5·2	0·754	0·5−0·4	+0·33
		F (*b*) 1610·5	6	1019·5	+16·0	1·987	0·1−0·05	+1·00
44	7·4	I 1731·3	4	52·8				
		F (*a*) 1720·3	3	338·7	−11·0	1·628	0·2−0·1	−0·64
		F (*b*) 1732·7	3	88·7	+1·4	0·309	0·8−0·7	+0·08
17	6·2	I 1890·7	3	660·7				
		F (*a*) 1905·3	3	2444·7	+14·6	0·767	0·5−0·4	+0·77
		F (*b*) 1903·3	3	714·7	+12·6	0·995	0·4−0·3	+0·67
9	6·6	I 5859·0	3	5774·0				
		F 5847·0	4	25860·0	−12·0	0·189	0·9−0·8	−0·25

The results are very clear-cut. With one exception, the differences between nitrogen contents before and after incubation are generally small and cannot be regarded as indicating a significant change in the nitrogen content of the soil, since the values of P are all above 0·05. The exception is represented by the acid soil No. 7, which showed a significant loss of nitrogen after 60 days. Apart from this single instance we have 68 cases where the change in nitrogen content does not exceed the analytical error; this is true irrespective of soil type, humus content, reaction, and presence or absence of *Azotobacter*. Even where the numbers of *Azotobacter* are highest (Nos. 8, 35 + $CaCO_3$, and 44) there is no indication of any gain of nitrogen (cf. de' Rossi, 1932*d*). The last column of the table shows that the changes vary between +2·41 and −3·11% of the original nitrogen content. These values, however, are uncommon, and quite small changes are the general rule. In Figure 4 the frequencies of the percentage changes are arranged in groups of 0·4%. This grouping reveals an approximately binominal distribution around a mean value of −0·192% with standard deviation ± 1·162. This approximate normality further strengthens the evidence that the changes are mainly due to accidental experimental errors. The fact that the mean is somewhat below zero might suggest a tendency of the soils to lose small quantities of

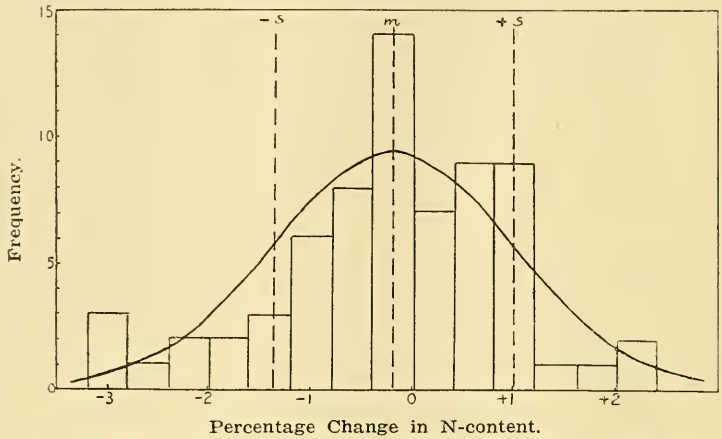

Fig. 4.—Distribution of percentage changes in nitrogen content of soils incubated without addition of organic matter (Table 7). *m*, mean; *s*, standard deviation. Theoretical curve of distribution constructed on the basis of Fisher's (1930) Table I.

nitrogen on incubation—losses which, although individually non-significant, might become significant in the aggregate. A closer inspection of the figures indicates that such small losses tend to be more common in definitely acid soils (pH 6·0 and less) than in the rest. In order to test whether this difference is apparent or real, the 68 percentage-values were divided into two groups (pH $\leqq$ 6·0, and pH $>$ 6·0), and the *t*-test of Fisher (1930, pp. 104–05) applied to decide whether the mean change in each group departs significantly from zero. For this test we calculate the standard deviation

$$s = \sqrt{\frac{S(x-\bar{x})^2}{n}}$$

$$\text{and } t = \frac{\bar{x}\sqrt{n+1}}{s}$$

The value of P is found, as before, from Fisher's Table iv. We have 40 data on soils of pH above 6·0; the calculation gives:

$n+1 = 40$. $\bar{x} = +0{\cdot}093$.
$s = \pm 1{\cdot}051$, $t = 0{\cdot}559$.
$n = 39$, P = 0·6–0·5 (not significant).

The same calculation on the 28 data on soil of pH 6·0 and less gives:

$n+1 = 28$. $x = -0{\cdot}56$.
$s = \pm 1{\cdot}237$, $t = 2{\cdot}395$.
$n = 27$, P = 0·05–0·02 (barely significant).

Although perhaps no very great importance should be attached to these figures, they show that in the faintly acid to alkaline soils the change in nitrogen content is insignificant, whereas in the definitely acid soils there is, if anything, an indication of small losses of nitrogen. That such a tendency to loss of nitrogen exists is also suggested by the fact that an unquestionably significant loss took place in the most acid soil (No. 7, pH 5·1) after prolonged incubation.

The numerous statements quoted above, concerning nitrogen fixation in soils incubated without addition of energy material, cannot thus in any way be confirmed in the case of the soils included in this series of experiments. When looking for an explanation for this contradiction, one cannot but notice that in most of the reports of gains of nitrogen under these conditions there are circumstances which make the reality of the gains appear doubtful. Firstly there is, as already mentioned, the possibility of an error due to analysis of dry soil before and moist soil after the incubation, as pointed out by Bal (1925). This, however, cannot offer a general explanation, since many authors state that dry soil was always analysed, and it is by no means all soils that show this effect (Olsen, 1937). Secondly there are many cases where the analytical error was not explicitly stated; although it is unlikely that this would be so severe as to cover changes of 10% of the nitrogen content or more, it may yet be an important contributing factor in producing fictitious gains of nitrogen, particularly if only two parallel determinations are made (Pfeiffer et al., 1906). Thirdly, some authors in the U.S.A. have used a modification of the Kjeldahl method (the "Official" method, as described in: Official and Tentative Methods of Analysis, by the Association of Official Agricultural Chemists, Ed. 5, 1935), in which the digestion is discontinued as soon as the acid is colourless, "or nearly so". In most other instances the time of digestion is not expressly stated, but it seems to be common practice to continue the heating only until the dark colour has disappeared (cf. Lemmermann, 1934). This is almost certainly insufficient to ensure complete conversion of the humus-nitrogen into ammonium sulphate, as shown by Christensen (1927), Ashton (1936), and Olsen (1937); in agreement herewith, Greaves and Greaves (1932) found that this procedure gave only 83% of the nitrogen found by the method of Dumas.[16] It might be concluded that changes in the proportion of nitrogen convertible into ammonium sulphate by short digestion might account for some of the apparent gains of nitrogen, which would automatically result if some of the humus nitrogen became more easily digestible during incubation of the soil. In order to test this possibility, 5 of the soils in Table 7 were re-analysed by the dry-digestion method with salicylic-sulphuric acid, digestion being stopped when the dark colour had gone (as in the "Official" method). The result are found in Table 8.

These nitrogen figures are all significantly lower than the corresponding figures found by "wet" digestion and prolonged heating. This is particularly noticeable in No. 26 (a heavy black loam; cf. Bal, 1925) and least marked in No. 44. In two cases, No. 29 and No. 44, there is actually a not very large, but still significant, difference in nitrogen content of soil before and after incubation. These differences would have impressed as actual gains of nitrogen if the soils had been analysed by this method only, but in view of the results in Table 7 they must be regarded as merely representing increases in the proportion of nitrogen convertible by short digestion (cf. Olsen, 1937, on the alleged nitrogen fixation by germinating seeds of leguminous plants).

Fourthly, in most of the experiments of Greaves (1914–16) and Greaves and Nelson (1923) the gains of nitrogen were reported as excess over sterile (presumably autoclaved) control soil. It has previously been shown, first by

[16] This method, indeed, may have a tendency to give too high values when applied to porous materials of a low nitrogen content like soil, as pointed out by Berthelot (1889); if it were not for this limitation, it would be highly desirable that this method should be used in exact experiments on nitrogen fixation in the soil.

TABLE 8.

Nitrogen-determination in Soils by Short-time Digestion.

Soil No.	Total N, p.p.m., Mean ($\bar{x}$).	n+1.	S $(x-\bar{x})^2$.	Gain (+) or Loss (−) p.p.m. ($\bar{x}_2-\bar{x}_1$).	t.	P.	Recovery of N in % of "wet". Digestion (Cf. Table 7.)
34. Before incubation	660·3	3	660·3	—	—	—	88·8
Inc. 30 d.* ..	664·7	3	368·7	+4·4	0·300	0·8−0·7	89·2
51+52. Before incubation ..	754·5	2	12·5	—	—	—	92·5
Inc. 30 d. ..	751·0	2	32·0	−3·5	0·742	0·6−0·5	91·8
29. Before incubation	855·3	4	452·8	—	—	—	88·3
Inc. 30 d. ..	881·0	4	14·0	*+25·7*	4·124	*<0·01*	91·7
26. Before incubation	1008·7	3	554·7	—	—	—	79·3
Inc. 30 d. ..	999·7	3	448·7	−9·0	0·696	0·6−0·5	79·3
44. Before incubation ..	1641·0	4	270·0	—	—	—	94·8
Inc. 30 d. ..	1669·5	4	637·0	*+28·5*	3·278	*0·02−0·01*	96·7

* Mixtures of equal parts of samples (*a*) and (*b*) in Table 7.

TABLE 9.

Influence of Sterilization and Subsequent Incubation on Nitrogen Content of Soils.

Soil No. and Treatment.	Total N Mean, p.p.m. ($\bar{x}$).	n+1.	$S(x-\bar{x})^2$.	Gain (+) or Loss (−) of N, p.p.m. due to		t.	P.
				Sterilization.	Incubation.		
15 (pH 5·4):							
Untreated ..	755·2	9	293·6	—	—	—	—
Autoclaved ..	725·7	3	162·7	*−29·5*	—	6·551	*<0·01*
Incubated ..	724·0	3	14·0	—	−1·7	0·460	0·7−0·6
28 (pH 5·7):							
Untreated ..	1576·0	10	1654·0	—	—	—	—
Autoclaved ..	1526·7	3	320·6	*−49·3*	—	6·077	*<0·01*
Incubated ..	1533·0	2	128·0	—	+6·3	0·564	0·7−0·6
64 (pH 6·4):							
Untreated ..	1822·0	3	182·0	—	—	—	—
Autoclaved ..	1825·3	3	130·7	+3·3	—	0·457	0·7−0·6
Incubated ..	1828·3	3	52·7	—	+3·0	0·543	0·7−0·6
30 (pH 7·7):							
Untreated ..	1071·7	10	1334·1	—	—	—	—
Autoclaved ..	1069·0	3	126·0	−2·7	—	0·316	0·8−0·7
Incubated ..	1032·0	3	152·0	—	*−39·7*	5·189	*<0·01*

Berthelot (1888), later by Pfeiffer et al. (1906) and Warmbold (1908), that sterilization as well as incubation of sterile soil may result in losses of nitrogen, which might represent still another source of error. A few experiments in this direction were carried out on 4 soils in Table 2. Portions of 50 gm. of moist soil were placed in 100 c.c. Erlenmeyer flasks and autoclaved for 45 minutes at 20 lb. pressure. One portion was analysed for total nitrogen immediately after the treatment, and another was analysed after incubation for 4 weeks at 28–30°C. Tests on nutrient agar slopes showed all the samples to be sterile both before and after incubation. The results are seen in Table 9. (It may here be remarked that the figures for "untreated" soil, except No. 64, are based on the total number of nitrogen determinations before and after incubation in Table 7; this must be considered permissible, since the changes in nitrogen content were never significant.)

The two strongly-acid soils, Nos. 15 and 28, lost significant quantities of nitrogen (3–4%) by autoclaving, but remained unaltered during incubation. The alkaline soil No. 30 was not affected by the autoclaving, but lost about 4% of its nitrogen during incubation, and finally the faintly-acid soil No. 64 remained unchanged by both sterilization and incubation. The loss of nitrogen as a consequence of sterilization is thus a common phenomenon, and if overlooked it may easily create the false impression of a gain of nitrogen in non-sterilized soil. For this reason too, as mentioned in the introduction, the statements of Greaves and co-workers (1928–32) on nitrogen fixation by numerous micro-organisms in sterilized soil cannot be accepted as valid, until it has been proved that the sterile control soil did not, like soil No. 30 in the present experiment, lose nitrogen on incubation.

The fact that spurious increases in the nitrogen content may be caused by incomplete conversion of the humus nitrogen into ammonia due to an insufficiently long digestion time as well as by the use of sterilized soil as control, combined in many cases with lack of information on the analytical error and uncertainty as to whether dry or moist soil was analysed, thus leads us to the conclusion that the occurrence of a measurable nitrogen fixation in soils incubated without addition of energy material must still be regarded as not proved with absolute certainty. Such claims can be accepted only when all the possible sources of error mentioned have been ruled out; this cannot be said to be the case in any of the experiments reviewed here, even in those that appear best documented (Remy, 1909, in whose data the occasional gains of nitrogen in soil with addition of $HgCl_2$ also suggest the operation of still other factors).

Besides this, the theoretical difficulties of explaining an intensive nitrogen fixation under these conditions are great. To take an example: in pure cultures of *Azotobacter* the yield of fixed nitrogen rarely, if ever, exceeds 1 part of nitrogen fixed per 16 parts of carbon consumed (= 25 mgm. N per gm. of glucose). If an ordinary soil containing 1% organic carbon gain only 50 p.p.m. nitrogen through the activity of *Azotobacter*, this will imply a consumption of *at least* 800 p.p.m. carbon, or 8% of the total carbon-content of the soil, by *Azotobacter* alone (cf. the estimate of Remy, 1909), which must derive the whole of its nitrogen supply from the atmosphere. In normal soils, however, the organic matter has a C/N-ratio in the neighbourhood of 10:1 (Waksman, 1932), and its decomposition is accompanied by production of ammonia and/or nitrate. It is difficult to see how this could be reconciled with a maximal nitrogen-fixing efficiency of *Azotobacter*, even if presence of molybdenum or vanadium compounds in the

soil might allow some fixation in the presence of nitrate (Bortels, 1936); in this connection it is interesting to note that Engel (1931*b*) found a C/N-ratio wider than 20:1 necessary for nitrogen fixation, and the depressing influence of nitrate on the development of *Azotobacter* in the soil is well known (Winogradsky, 1926). Further, we have assumed that the carbon compounds utilized by *Azotobacter* had the same value as glucose; it is extremely doubtful whether this assumption is justified, since the bulk of the soil "humus" is inaccessible to *Azotobacter*, which has to thrive on the organic by-products of the metabolism of other organisms. These by-products are likely to be mostly simple alcohols and organic acids (Winogradsky, 1932) of a lower energetic value per unit of carbon than glucose (Mockeridge, 1915; Gainey, 1928). It is thus necessary to strain the evidence badly in order to account for a nitrogen fixation of the order of some 5% of the total nitrogen content of the soil, unless we postulate *either* that *Azotobacter* functions in the soil in a manner totally different from that in pure cultures, *or* that unknown organisms with different properties act as nitrogen-fixers, none of which postulates is supported by experimental evidence.

These considerations, together with the experimental results, justify us in concluding that the claims of particularly vigorous processes of nitrogen fixation in soils from arid climates appear groundless so far as they are based on experiments of the type discussed here.

But even if wheat soils show no measurable increase in nitrogen content when incubated under conditions corresponding to those of bare-fallowing in summer time with good conservation of moisture, our problem is far from solved; we shall now turn to the question of the potential nitrogen-fixing capacity of the soils, as it is exhibited when an excess of directly available energy material is added.

2. *Soils with Addition of Glucose.*

That nitrogen fixation in soil may be stimulated to a marked extent by addition of directly assimilable carbonaceous food material, such as mannite or glucose, was shown first by Schneider (1906) and Koch et al. (1907), who found that up to 10–12 mgm. nitrogen could be fixed per gm. of glucose added to the soil. These investigations have been followed by a large number of contributions, which need not be reviewed in detail. Many of the authors quoted in the previous chapter have carried out experiments with addition of sugar to the soil, besides the experiments on soils incubated without addition of energy material. We may mention the papers of Remy (1909), Lemmermann and Blanck (1908), Lemmermann et al. (1910), Marr (1910), Koch (1909), Walton (1915), Greaves (1916), Traaen (1916), Hills (1918), Neller (1920), Greaves and Carter (1920), Meggitt (1923), Lander and Ali (1925), Zoond (1926), Christensen (1927), de' Rossi (1932*d*), Lemmermann and Themlitz (1934), Engel (1934), and Dhar (1937). Most of these investigations have shown that the maximal yield of fixed nitrogen under conditions favourable for the development of *Azotobacter* (good aeration, suitable temperature, neutral to faintly alkaline reaction, adequate supply of mineral nutrients, esp. phosphate) does not materially exceed 10–12 mgm. N per gm. of sugar or mannite. Statements of higher gains are comparatively few and not always convincing. Observations of this kind have been made by Brown and Smith (1912) in Iowa (in one instance a gain corresponding to 102 mgm. N per gm. mannite; misprint?), Greaves (1914) in Utah (up to 34 mgm. N per gm. mannite), Waksman and Karunakar (1924) in New Jersey (in

one instance 45 mgm. N per gm. mannite), Burgess (1932) in California (up to 27 mgm. N per gm. mannite), Turk (1936) in Michigan (up to 19 mgm. N per gm. mannite), and Bortels (1937) in Germany; the last author gives figures suggesting gains of 50–60 mgm. N per gm. glucose. It applies to all these statements, that the analytical procedure and the experimental error have not been reported in detail, and it is therefore entirely possible that some of the sources of error discussed in the previous chapter may have made some of the gains appear unduly high. It is noteworthy that Waksman and Karunakar, among whose data such a case appears, do not attach any great importance to these figures. Thus even the data from California, Utah, Iowa and Michigan cannot be said to prove that nitrogen fixation is more intensive in the soils from these arid or semi-arid districts than elsewhere.

Most of the research work in this direction has been of a chiefly chemical nature. Where bacteriological investigations have been carried out, they have usually shown that gains of nitrogen under aerobic conditions are associated with development of *Azotobacter*; this was first shown by Schneider (1906), Koch et al. (1907), and Remy (1909). Koch (1909) observed no gain of nitrogen in soils not containing *Azotobacter*, and in agreement herewith Waksman and Karunakar (1924) found no fixation in soil of pH below 6·0. Winogradsky (1925–26) showed that addition of assimilable carbon compounds results under favourable soil conditions in such a multiplication of *Azotobacter* that this organism altogether dominates the microscopic picture of the soil flora. He also observed that increasing concentrations of nitrate tend to counteract the development of *Azotobacter* by enabling other organisms to compete for the available energy material. No nitrogen determinations were made directly on the soils, but the "spontaneous cultures" of *Azotobacter* on silica-gel plates (Winogradsky, 1926–28) did not fix more than 10–12 mgm. N per gm. mannite; without development of *Azotobacter* there was no significant gain of nitrogen in these cultures, which led Winogradsky to the conclusion that this organism was alone responsible for nitrogen fixation in soil under aerobic conditions. A few authors (De and Pain, 1936; Turk, 1936) have reported nitrogen fixation in soils of acid reaction and said to be free from *Azotobacter*; since similar gains were observed in the same soils without addition of organic matter, these statements cannot be accepted as convincing.

Actual counts of *Azotobacter* in connection with nitrogen fixation experiments of this type have only been carried out in a few cases. The first step in this direction (apart from the semi-quantitative tests on lime plaques by Schneider, 1906, and Remy, 1909) seems to have been made by Hills (1918), who studied the influence of nitrate on cell multiplication and nitrogen fixation by *Azotobacter*— in both sterilized and untreated soil. Hills' results, however, have only a limited value, partly because the *Azotobacter*-counts and the nitrogen determinations were carried out in separate experimental series, but especially because the nitrogen figures are obviously faulty (as also pointed out by Zoond, 1926), owing to the use of dilute salicylic-sulphuric acid for the analysis, and consequent loss of NO_3–N (cf. Bristol and Page, 1923).[17] de' Rossi (1932*d*) carried out some highly interesting experiments, which showed that gains of 0·5–1·0 mgm. nitrogen in soil with addition of 2% mannite after 10–12 days at 32°C. were accompanied by the presence of 200 to 770 mill. *Azotobacter* (by plate count) at the end of the

[17] The loss of nitrogen in Hills' experiments thus need not be due to the physiological activity of *Azotobacter*, as suggested by Bonazzi (1921).

experiment. Although the data were few, there was some evidence of a correlation between amounts of nitrogen fixed and numbers of *Azotobacter*:

	mgm. N fixed in 5 gm. of soil.	*Azotobacter*, mill. per 5 gm. of soil.
Exp. IV, 5–	0·4 ..	373·9
,, IV, 7–	0·5 ..	401·9
,, V, 3–	0·5 ..	203·6
,, VI, 2–	1·0 ..	772·3

A remarkable series of investigations has been carried out in recent years in India by Dhar and co-workers (summarized by Dhar, 1937), who claim to have found evidence of a purely photochemical fixation of nitrogen; this process was stated to require a consumption of energy material similar to that in the biological fixation. In the latest series of experiments, Dhar (1937) compared soil samples with addition of various organic compounds, placed in sunlight and in the dark; periodical plate counts of *Azotobacter* and determinations of nitrogen and carbon were carried out; but neither the method of counting nor the analytical methods were clearly described. The counts of *Azotobacter* were regularly higher in soils incubated in darkness, where they often rose to several hundred millions per gm., but in soils exposed to sunlight the gains of nitrogen per unit of carbon disappeared from the soil were generally 50 to 100% higher than in the corresponding experiments in darkness. Dhar concludes from these results that "the nitrogen fixation in soils mixed with energy-rich materials is approximately 50% bacterial and 50% photochemical". This argument, however, seems altogether unconvincing. It is particularly important to notice that even the highest gain of nitrogen reported (15·7 mgm. per gm. of oxidized saccharose-carbon in soil exposed to sunlight for 3½ months) merely corresponds to a fixation of about 7 mgm. N per gm. of sugar consumed (or more precisely, converted into carbon dioxide and water; somewhat more must have been used for the production of bacterial substance); this could easily have been achieved by *Azotobacter* alone. That the counts of *Azotobacter* were lower under exposure to sunlight may find simple explanation in the entirely different temperature conditions; the soils in darkness were incubated at the optimal temperature of 28–33°C., whereas in the exposed soils the temperature must have fluctuated greatly and was sometimes reported to be as high as 48°C. It is highly probable that the recurrence of such high temperatures, together with the possible direct lethal action of the sunlight, would greatly accelerate the death-rate of the *Azotobacter*-cells and thereby prevent them from accumulating to the extent observed in the darkness-experiments, while renewed multiplication of *Azotobacter* might set in at night-time, and generally under less unfavourable temperature conditions; as an analogy, one might quote the experiments of Cutler and Bal (1926), who found that protozoa reduced the numbers of *Azotobacter* in solution cultures, but stimulated the rate of nitrogen fixation, presumably by decimating the bacterial population and thereby stimulating the production of new cells. Further, the numbers of *Azotobacter* stated to be present in the original soil were so high—2 to 12 mill. per gm.—that one might well doubt whether they were really all *Azotobacter* (cf. Dhar and Seshacharyulu (1936), as mentioned previously). Until more rigid proof is forthcoming, one cannot therefore regard the existence of photochemical nitrogen fixation as proved; and even if it were so, it does not appear from Dhar's data that the process would be more economical than the fixation by *Azotobacter*, so far as the consumption of energy material is concerned.

Nitrogen fixation experiments with sugar, etc., under anaerobic conditions have been comparatively few in number. The fixation is generally found to be less vigorous than under aerobic conditions, although it may reach some 5 to 6 mgm. nitrogen per gm. of sugar or mannite supplied, as shown by Traaen (1916), Greaves and Carter (1920), Turk (1936), and others quoted by Waksman (1932). The anaerobic conditions have mostly been produced simply by addition of an excess of water to the soil. Even if the saturation is not complete, the addition of sugar gives rise to an abundant development of vegetative clostridia, as shown by Winogradsky (1925–26), but on the other hand *Azotobacter* may still develop in fully water-saturated soil (Traaen, 1916).

A number of soils included in the present investigation were tested for nitrogen fixation on addition of glucose, chiefly under aerobic conditions. In the first series of experiments 16 soils from Table 2 were examined; mostly soils with a nitrogen content of less than 0·1% were selected, in order to be able to detect small changes with more certainty. The experiments were arranged exactly in the same manner as those without addition of organic matter (Table 7), and were carried out simultaneously with these. Altogether 2·5% of pure glucose[18] were added to the soil, 1·5% at start and 1·0% after 15 days. After 30 days' incubation at 28–30°C. the soil was air-dried and analysed for total nitrogen; some time, normally 4 to 5 days, after each addition of sugar, samples were taken for total microscopic counts of bacteria and for plate counts of *Azotobacter*; in some of the earlier experiments, before the plate method had been adopted in its final form, a loopful of soil suspension was simply streaked out on dextrine agar, or a number of soil particles were planted on silica-gel with mannite (Winogradsky, 1925).

Table 10 shows the results of the nitrogen determinations. As shown in the previous series of experiments, the nitrogen content of the soils never changed significantly during incubation without organic matter for 30 days, i.e. all determinations on the same soil before and after incubation may be regarded as belonging to the same population; therefore, the average of all these determinations may, as in Table 9, be regarded as the control with which the nitrogen content of the soil with glucose addition is to be compared. It is these general averages from the figures in Table 7 that are here given as "initial" nitrogen contents. Since these are based on nine or more parallel determinations, we get a considerably higher accuracy than by using only the actual initial nitrogen figures in Table 7. The significance of the differences was found as in the previous series, by means of the *t*-test.

Only soil No. 8, which is outside the wheat district, has pH 7·4 and had received an extra application of 0·2% $CaHPO_4$, shows a considerable gain of nitrogen. Although this gain appears large in proportion to the original nitrogen content (about one-third), it is actually only moderate, since it corresponds to a fixation of about 6 mgm. N per gm. of glucose added. In soil No. 25, a moderately productive wheat soil of pH 6·5, there is in one of the parallel dishes a small gain of nitrogen, equivalent to 10% of the original nitrogen content, or a fixation of 2·3 mgm. N per gm. of glucose. All other soils, irrespective of type, reaction, manurial treatment and presence or absence of *Azotobacter*, have entirely failed to fix nitrogen. Indeed, the addition of glucose has in many cases resulted in a significant loss of nitrogen; it is to be remembered that if some of the glucose

[18] On the basis of air-dry soil.

TABLE 10.

Nitrogen-fixation in Soils with Addition of 2·5 (=1·5+1·0)% Glucose.

Soil No.	Total N p.p.m., Mean ($\bar{x}$).	n+1.	S $(x-\bar{x})^2$.	Gain (+) or Loss (−) of N, p.p.m., ($\bar{x}_2-\bar{x}_1$).	t	P
24. Initial	392·7	10	310·1	—	—	—
Inc. plus glucose (*a*) ..	398·8	3	204·7	+6·1	1·355	0·3−0·2
(*b*) ..	381·3	3	162·7	*−11·4*	2·902	*0·02−0·01*
8. Initial	446·8	11	804·0	—	—	—
Inc. plus glucose (*a*) ..	595·7	3	160·7	*+148·9*	—	—
(*b*) ..	602·5	4	711·0	*+155·7*	—	—
19. Initial	455·2	9	315·2	—	—	—
Inc. plus glucose (*a*) ..	449·3	3	2·7	−5·9	1·570	0·2−0·1
(*b*) ..	453·7	3	12·7	−1·5	0·393	0·8−0·7
25. Initial	535·7	11	980·6	—	—	—
Inc. plus glucose (*a*) ..	592·7	3	448·7	*+57·0*	6·369	*<0·01*
(*b*) ..	523·3	3	82·3	−12·4	2·023	0·1−0·05
21. Initial	582·1	10	391·7	—	—	—
Inc. plus glucose (*a*) ..	586·7	3	4·7	+4·6	1·164	0·3−0·2
(*b*) ..	580·7	3	20·7	−1·4	0·347	0·8−0·7
10. Initial	631·9	10	1038·1	—	—	—
Inc. plus glucose (*a*) ..	633·0	4	654·0	+1·1	0·157	0·9−0·8
(*b*) ..	608·0	4	854·0	*−23·9*	3·217	*<0·01*
11. Initial	634·1	14	2718·6	—	—	—
Inc. plus glucose (*a*) ..	633·8	4	1332·6	−0·3	0·026	1·0−0·9
(*b*) ..	631·7	3	164·7	−2·4	0·272	0·8−0·7
14. Initial	649·3	12	2361·5	—	—	—
Inc. plus glucose (*a*) ..	639·7	3	40·7	−9·6	1·094	0·3−0·2
(*b*) ..	637·0	3	104·0	−12·3	1·384	0·2−0·1
20. Initial	653·1	11	1394·4	—	—	—
Inc. plus glucose (*a*) ..	638·3	3	138·7	−14·8	2·011	0·1−0·05
(*b*) ..	661·8	3	332·6	+8·7	1·114	0·3−0·2
7. Initial	731·8	11	4101·6	—	—	—
Inc. plus glucose (*a*) ..	699·0	4	1834·0	*−32·8*	2·689	*0·02−0·01*
(*b*) ..	713·7	3	1164·7	−18·1	1·326	0·3−0·2
23. Initial	748·7	10	892·9	—	—	—
Inc. plus glucose (*a*) ..	729·7	3	50·7	*−19·0*	3·116	*<0·01*
(*b*) ..	728·7	3	460·7	*−20·0*	2·735	*0·02−0·01*
15. Initial	755·2	9	293·6	—	—	—
Inc. plus glucose (*a*) ..	735·3	3	194·7	*−19·9*	4·933	*<0·01*
(*b*) ..	734·3	3	48·7	*−20·9*	6·182	*<0·01*
1. Initial	797·5	6	695·5	—	—	—
Inc. plus glucose ..	789·7	3	658·7	−7·8	0·792	0·5−0·4
6. Initial	827·1	11	2528·8	—	—	—
Inc. plus glucose (*a*) ..	801·3	3	620·6	*−25·8*	2·445	*0·05−0·02*
(*b*) ..	778·3	3	768·7	*−48·8*	4·520	*<0·01*

30. Initial	1071·7	10	1334·1	—	—	—
Inc. plus glucose (*a*) ..	1076·0	3	344·0	+4·3	0·529	0·7—0·6
(*b*) ..	1064·8	4	802·6	−6·9	0·874	0·4—0·3
2. Initial	1256·8	6	2004·6	—	—	—
Inc. plus glucose ..	1259·7	3	930·7	+2·9	0·200	0·9—0·8

Appendix: Soils with addition of Na_2MoO_4 or Na_2HPO_4 besides 2·0% glucose. Inc. 28 days.

10. Initial	631·9	10	1038·1	—	—	—
Inc. plus glucose alone ..	613·3	3	68·7	*−18·6*	2·817	*0·02—0·01*
Do. plus Na_2MoO_4 (*a*) ..	628·7	3	40·7	−3·2	0·491	0·7—0·6
(*b*) ..	622·7	3	74·7	−9·2	1·390	0·2—0·1
Do. plus Na_2HPO_4 (*a*) ..	672·3	3	162·7	*+40·4*	5·874	*<0·01*
(*b*) ..	663·3	3	164·7	*+31·4*	4·562	*<0·01*
30. Initial	1071·7	10	1334·1	—	—	—
Inc. plus glucose alone ..	1050·0	3	1178·0	*−21·7*	2·325	*0·05—0·02*
Do. plus Na_2MoO_4 (*a*) ..	1060·7	3	384·7	−11·0	1·471	0·2—0·1
(*b*) ..	1040·7	3	50·7	*−31·0*	4·197	*<0·01*
Do. plus Na_2HPO_4 (*a*) ..	1050·3	3	62·0	*−21·4*	2·887	*0·02—0·01*
(*b*) ..	1033·3	3	98·7	*−38·4*	5·110	*<0·01*

or its decomposition products persisted in the soil after incubation, a certain decrease in the nitrogen content would automatically result from the "dilution" of the soil with glucose. If no glucose at all were broken down to carbon dioxide and water (which of course will not happen), the resulting decrease would be 2·5% of the original nitrogen content, and this is frequently seen to be exceeded. It is noteworthy that such a loss has taken place also in the highly fertile, alkaline soil No. 30.

The microbiological examinations, recorded in Table 11, show a somewhat more complicated picture. The cultural tests show complete absence of *Azotobacter* in all soils of pH 6·0 and less (Nos. 19, 21, 14, 7, 15, 6), and even in the alkaline soils No. 23 and 24. Plates from the first of these showed a large number (about 2·5 mill. per gm.) of a fungus closely resembling *Dematium pullulans*, which has been claimed to fix nitrogen by Löhnis and Pillai (1908). This fungus and several other organisms from this experimental series were tested for nitrogen fixation with a negative result (Table 4). Soils No. 8, 10, and 11 show abundant growth of *Azotobacter* by qualitative tests. Plate counts show that in soil No. 25 the numbers of *Azotobacter* have risen to several millions per gm., highest in the dish where nitrogen was fixed, and even higher numbers are reached in soil No. 30, although no nitrogen was fixed here. The cultural tests thus merely show that nitrogen fixation coincides with multiplication of *Azotobacter*, which on the other hand may multiply to the order of many millions per gm. without any nitrogen fixation taking place.

The direct microscopic counts show some interesting features. A certain number of large globular organisms, 1·2–2·0μ in diameter, resembling *Azotobacter*-cells, were seen in practically all soils, but rarely in numbers exceeding some 4 to 5% of the total microflora. The only exceptions from this rule are represented by the soils where nitrogen fixation took place, viz., No. 8 and one of the parallels of No. 25, particularly the former, where the *Azotobacter*-like organisms occupy quite a dominant position, and where the fixation was most intense. Otherwise the microscopic flora appeared rather uncharacteristic; like the normal soil microflora (Thornton and Gray, 1934) it was composed mainly of small, short, non-

TABLE 11.

Microbiological Examination of Soils with Addition of Glucose.

Soil No. and Time of Examination.	Total Bacteria Direct Count. Mill. per gm.	*Azotobacter*-like Organisms. % of Total Bacteria.	*Azotobacter*-like Organisms. Mill per gm.	*Azotobacter* by Cultural Methods.	N Fixed, Mgm. per gm. Glucose.
24. Initial	—	—	—	M.S.: present.	—
Incubated:					
(*a*) 6 d. ..	577±51·0	2·0	(12)	D.A.: absent; after 6 d.:	Nil.
21 d. ..	482±52·0	3·1	(15)	numerous white bacterial	
(*b*) 6 d. ..	750±68·3	1·4	(11)	colonies; 21 d:	Nil.
21 d. ..	596±51·5	1·3	(8)	numerous fungi.	
8. Initial	—	—	—	D.A.: 9 per gm.	
Incubated:					
(*a*) 4 d. ..	—	—	—	D.A. and S.G.: abundant.	6·2
18 d. ..	4236±148	44·9	1900±112	Colonies of other	
(*b*) 4 d. ..	1770±90·1	21·2	353±37·4	organisms not con-	5·9
18 d. ..	3895±198	28·0	1090±81·8	spicuous.	
19. Initial	—	—	—	Absent.	—
Incubated:					
(*a*) 5 d. ..	1140±65·0	0·9	(10)	D.A.: absent; numerous	Nil.
20 d.	1303±82·5	0·5	(7)	fungi and spore-forming	Nil.
(*b*) 20 d. ..	1301±86·4	0·6	(8)	bacilli.	
25. Initial	—	—	—	M.S.: present.	—
Incubated:					
(*a*) 5 d. ..	2058±125	8·0	166±28·9	D.A.: 1·4 mill. per gm.	2·3
18 d. ..	3495±194	14·5	518±56·9	12·5	
(*b*) 7 d. ..	2303±147	5·9	137±28·1	7·0	Nil.
18 d. ..	1415±120	2·3	(33)	Absent.	
21. Initial	—	—	—	Absent.	—
Incubated:					
(*a*) 21 d. ..	—	—	—	D.A.: absent; numerous	Nil.
(*b*) 21 d. ..	—	—	—	fungi.	Nil.
10. Initial	—	—	—	D.A.: 20 per gm.	
Incubated:					
(*a*) 4 d. ..	1647±95·6	1·7	(27)	D.A. and S.G.: present;	Nil.
(*b*) 4 d. ..	1756±96·6	1·1	(19)	fungi numerous.	
11. Initial	—	—	—	M.S.: present.	—
Incubated					
(*a*) 4 d. ..	—	—	—	D.A. and S.G.: present;	Nil.
(*b*) 4 d. ..	1368±76·9	3·5	(58)	fungi numerous.	Nil.
14. Initial	—	—	—	Absent.	—
Incubated:					
(*a*) 5 d. ..	1634±114	1·2	(20)	D.A. and S.G.: absent;	Nil.
(*b*) 5 d. ..	1333± 89	1·8	(24)	numerous fungi and spore-formers.	Nil.
20. Initial	—	—	—	Absent.	
Incubated:					
(*a*) 4 d. ..	—	—	—	D.A.: absent; numerous	Nil.
(*b*) 4 d. ..	—	—	—	fungi.	Nil.

23. Initial	—	—	—	M.S.: present.	
Incubated:				D.A.: absent; numerous	
(*a*) 8 d. ..	553±46·4	1·8	(10)	fungi, *Penicillium*, *De-*	Nil.
21 d. ..	1267±82·4	3·4	(46)	*matium* (?), after 21 d.	
(*b*) 21 d. ..	540±45·0	1·2	(6)	*Trichoderma*.	Nil.
7. Initial	—	—	—	Absent.	
Incubated:				D.A. and S.G.: absent;	
(*a*) 6 d. ..	554±56·0	(0)	(0)	many fungi (*Penicillium*).	Nil.
(*b*) 6 d. ..	496±54·6	0·9	(4)		Nil.
15. Initial	—	—	—	M.S.: present.	
Incubated:					
(*a*) 5 d. ..	1673±111	1·5	(25)	D.A.: absent; numerous	Nil.
(*b*) 5 d. ..	1825±101	1·8	(33)	fungi, some big spore-formers.	Nil.
6. Initial	—	—	—	Absent.	
Incubated:				D.A. and S.G.: absent;	
(*a*) 6 d. ..	1094±94·9	3·1	(34)	many fungi and big	Nil.
(*b*) 6 d. ..	1092±83·0	4·9	(54)	spore-formers.	Nil.
30. Initial	—	—	—	M.S.: present.	
Incubated:					
(*a*) 5 d. ..	2297±177	7·2	166±38·8	D.A.: 2·1 mill. per gm.	Nil.
21 d. ..	3295±200	7·3	182±31·1	21·4	
(*b*) 5 d. ..	1883±140	2·3	(43)	2·7	Nil.
21 d. ..	3308±201	3·5	113±27·8	23·6	

Appendix: Soils with addition of Na_2MoO_4 or Na_2HPO_4 besides 2·0% glucose.

10. Glucose alone:					
5 d. ..	1038±68·5	2·2	(23)	D.A.: 1·9 mill. per gm.	Nil.
19 d. ..	483±47·9	4·2	(20)	absent.*	
Do. plus Na_2MoO_4:					
(*a*) 5 d.	1544±99·1	5·2	80·3±18·3	D.A.: >0·2 mill.	Nil.
19 d.	752±57·0	1·3	(10)	absent.*	
(*b*) 5 d.	1508±74·3	2·5	(38)	1·0 mill.	Nil.
19 d.	749±60·4	2·1	(16)	absent.*	
Do. plus Na_2HPO_4:					
(*a*) 4 d.	2476±163	16·9	370±47·8	D.A.: 174 mill.	2·0
28 d.	843±60·3	22·5	187±31·5	3·6 mill.	
(*b*) 4 d... ..	1494±97·9	32·8	487±50·2	>200 mill.	1·6
28 d.	928±62·8	11·0	107±19·6	absent.*	
30. Glucose alone:					
4 d.	1550±116	1·2	(19)	D.A.: 0·13 mill.	Nil.
18 d.	2419±153	5·3	127±26·9	37·1 mill.	
Do. plus Na_2MoO_4:					
(*a*) 4 d.	2641±149	1·1	(29)	D.A. 0·40 mill.	Nil.
18 d.	2109±147	5·3	114±27·3	35·2 mill.	
(*b*) 4 d.	—	—	—	0·17 mill.	Nil.
18 d.	1709±118	7·5	127±26·4	35·6 mill.	
Do. plus Na_2HPO_4:					
(*a*) 6 d.	1829±134	1·0	(18)	D.A.: 4·5 mill.	Nil.
32 d.	2494±195	6·2	156±37·6	57·0 mill.	
(*b*) 6 d.	1465±106	0·7	(10)	4·0 mill.	Nil.
32 d.	4333±268	5·1	218±39·9	51·5 mill.	

* All plates covered with green *Trichoderma*.

Abbreviations: D.A.: dextrine agar. M.S.: Beijerinck's mannite solution. S.G.: silicic acid gel plus mannite.

spore-forming rods and coccoid organisms, with occasional admixtures of larger rod-shaped bacteria, and mycelium and spores of fungi and actinomycetes. Some vegetative clostridia were seen in soil No. 25; otherwise these organisms were seen only very sporadically or mostly not at all.

It is somewhat surprising that no nitrogen fixation and no very conspicuous microscopic development of *Azotobacter* should take place in soils like Nos. 10, 11, 23, 24, and 30, of pH 6·4 to 7·7. A supplementary experiment was carried out with soils No. 10 and 30, in order to test whether this might be due to deficiency of phosphate or molybdenum. This experiment was carried out like the others, but with only 2% glucose (1% at start and 1% after 2 weeks), and with extra addition of: (*a*) nothing; (*b*) Na_2MoO_4 (No. 10: 13·3 p.p.m.; No. 30: 22·0 p.p.m.); (*c*) Na_2HPO_4 (No. 10: 0·03%; No. 30: 0·025%). The soils were incubated for 4 weeks, except No. 30 plus phosphate, which, due to external circumstances, was kept for 32 days. The results are given in the appendices to Tables 10 and 11.

The control soils with no addition besides glucose show exactly the same result as in the previous series: considerable multiplication of *Azotobacter* (by plate count), but no gain of nitrogen. Addition of molybdate affects neither the *Azotobacter*-counts nor the nitrogen-figures significantly; the lack of fixation thus does not appear to be due to molybdenum deficiency. In soil No. 10 the addition of phosphate stimulates the development of *Azotobacter* very markedly, as shown by both the direct and the plate counts, and a significant gain of nitrogen takes place, corresponding, however, to a fixation of only 1·6–2·0 mgm. N per gm. of glucose. In comparison herewith it is surprising to see that in soil No. 30 the addition of phosphate has only stimulated the multiplication of *Azotobacter* slightly and has not resulted in any nitrogen fixation (indeed, there is a significant loss of nitrogen). Some still unknown inhibitory factor seems to be operating here;[19] it is perhaps of some significance that that strain of *Azotobacter* which proved ineffective when first tested for nitrogen fixing capacity (Table 4), was isolated from this soil.

It is a highly important fact that *Azotobacter* may multiply to the extent of several millions per gm. of soil (by plate counting) without causing any measurable gain of nitrogen. In comparison with such numbers the few thousand *Azotobacter* per gm., which usually represent the maximum under natural soil conditions, would appear altogether insignificant (cf. de' Rossi, 1932*c*). Things might indeed be different, if the colonies in counts from natural soil should arise, not from individual cells, but from aggregates of thousands or even millions of cells. This, however, is hardly the case; in shaken soil suspensions the *Azotobacter*-like cells certainly may appear in clumps, but these are not normally of an extraordinary size (Winogradsky, 1925; Thornton and Gray, 1934), and the same even seems to be the case in undisturbed soil as shown by the contact-slide method (Rossi et al., 1936;[20] Starkey, 1938); moreover, these cells are not necessarily all *Azotobacter* (cf. Dianowa and Woroschilowa, 1931; Rossi et al., 1936). If the multiplication of *Azotobacter* is stimulated by addition of assimilable organic matter, most cells are found to occur singly or in very small aggregates (Winogradsky, 1926); this was also the case in the present experiments, as

[19] It can hardly be a question of the "weather-factor" (Stapp and Bortels, 1936), since this experiment was carried out simultaneously with the same on soil No. 10, in which nitrogen fixation took place.

[20] According to these authors, the bacterial "clusters" rarely contain more than about 30 individuals.

shown by the characteristic photographs (Pl. i, figs. 3–5). A comparison between the plate counts of *Azotobacter* and the corresponding direct counts of *Azotobacter*-like cells, where the latter are sufficiently numerous to be counted with a reasonable accuracy (more than 80 mill. per gm.) shows the following relationship:

Soil No.	Plate Count. Mill. per gm.	Direct Count. Mill. per gm.	Plate Count in % of Direct Count.
25 *a*, 5 d.	1·4	166	0·8
18 d.	12·5	518	2·4
b, 5 d.	7·0	137	5·1
30 *a*, 5 d.	2·1	166	1·3
21 d.	21·4	182	11·7
b, 21 d.	23·6	113	20·9
10+Mo, *a*, 5 d.	0·2	80	0·25
$+P_2O_5$, *a*, 4 d.	174·0	370	47·0
28 d.	3·6	187	1·9
b, 4 d.	(>200·0)	487	(>40·0)
30 (sec. exp.), 18 d.	37·1	127	29·2
+Mo, *a*, 18 d.	35·2	114	30·9
b, 18 d.	35·6	127	28·0
$+P_2O_5$, *a*, 32 d.	57·0	156	36·5
b, 32 d.	51·5	218	23·6

Thus in 8 cases out of 15 the plate method shows figures from one-fifth to nearly one-half of the total number of *Azotobacter*-like cells. Since their numbers are highest where nitrogen fixation has taken place and where the plate method also shows high numbers of *Azotobacter*, and since the percentage of *Azotobacter*-cells capable of developing into colonies on agar may be as low as a few per cent. (Beijerinck, 1909), we may tentatively assert that high direct counts of *Azotobacter*-like cells in the soils here dealt with are represented chiefly by actual *Azotobacter*-organisms.

We can now make a rough estimate of the quantity of nitrogen contained in the cell-substance of these organisms. If we assume that the *Azotobacter*-cells have an average volume of $5\mu^3$ [21] and a specific gravity of 1, and contain 20% dry matter with 10% nitrogen (Burk and Lineweaver, 1930), 1000 mill. cells will represent 0·1 mgm. N. If we compare the quantities of nitrogen actually fixed with the estimated nitrogen-contents of the highest numbers of *Azotobacter*-like cells observed, we find the following:

Experiment No.	Highest Count of *Azotobacter*-like Cells. Mill. per gm.	Nitrogen in Cells, p.p.m. (Calc.).	Total Nitrogen Fixed. p.p.m.	Cell-N in % of Fixed Nitrogen.
8 *a*	1,900	190	149	128
8 *b*	1,090	109	156	70
25 *a*	518	52	57	91
10+phosphate *a*	370	37	40	93
b	487	49	31	158

[21] Unfortunately, it is impossible to measure the size of the living *Azotobacter*-cells in the soil. A number of measurements of these cells on the slides from soils No. 8, 10 and 25 showed an average diameter of 1·7-1·8μ, corresponding to a cell volume of 2·5-3·0μ^3. Some shrinking, however, is caused by the drying and staining. Some tests on pure cultures of *Azotobacter* showed that the same method of staining as used in the direct counts caused the cells to shrink to about 60-70% of their volume in a living condition.

There is here the same rough proportion between numbers of *Azotobacter* and gains of nitrogen as in the experiments of de' Rossi (1932*d*) and, considering the wide margins of error, the quantities of nitrogen in the *Azotobacter*-like cells are remarkably similar to the actual gains of nitrogen. This suggests strongly that where nitrogen fixation has taken place, it has consisted in a synthesis of *Azotobacter*-cells, i.e. the process is under these conditions "growth-bound" as in pure cultures.

Admittedly a certain proportion of the cell nitrogen may have been derived from the soil and not from the atmosphere, but against this must be set the nitrogen contained in those generations of *Azotobacter*-cells that have arisen and disappeared again before the time of counting, as well as possible subsequent generations—a quantity which we cannot estimate with any accuracy, since we do not know the rates of death and reproduction of *Azotobacter*-cells in the soil.

If the nitrogen-fixation consists in a simple synthesis of *Azotobacter*-cells, it is not surprising that this organism may multiply vigorously without causing any significant fixation; for instance, a production of 50 mill. *Azotobacter*-cells per gm. of soil, deriving all their nitrogen from the atmosphere, would give an increase of only 5 p.p.m. of nitrogen—a quantity difficult or impossible to detect in a soil of normal nitrogen content. The assimilation of combined soil nitrogen must also be reckoned with; every p.p.m. of nitrate- or ammonia-nitrogen taken up by *Azotobacter* would suffice for the production of about 10 mill. cells per gm. of soil.

All soils from these experiments were tested qualitatively for nitrate and ammonia at the termination of the experiment. Apart from one exceptional case, nitrate was always absent, but small quantities of ammonia-N (2–3 p.p.m. by quantitative determination) were sometimes found. The exception was represented by soil No. 8, which contained the following quantities in p.p.m.:

	NO_3–N.	NH_4–N.	Total.	Increase.
Before incubation ..	3·7	6·8	10·5	—
After incubation (*a*) ..	7·4	11·7	19·1	8·6
After incubation (*b*) ..	24·1	5·6	29·7	19·2

The increases correspond to 6 and 12% respectively of the amounts of nitrogen fixed (149 and 156 p.p.m.). Apparently the glucose has been used up very quickly, after which the fixed nitrogen may undergo a rapid mineralization, as shown by Engel (1934; cf. also Burk and Horner, 1936).

As a supplement to the nitrogen fixation experiments in Table 11 with addition of phosphate, a number of soils were tested qualitatively for their ability to support an abundant *Azotobacter*-flora with and without addition of phosphate. These experiments were carried out by the soil-plaque method of Winogradsky (1926): to 50 gm. of air-dry soil were added 5% starch, 1% $CaCO_3$, 5 c.c. 0·01% solution of Na_2MoO_4, and sufficient distilled water to give the mixture the consistency of a firm paste, which was moulded into a plaque with a smooth surface in an ordinary Petri dish; similar plaques were given an extra addition of 0·2% $CaHPO_4$. To ensure presence of *Azotobacter*, each plaque was inoculated with 0·5 c.c. of a thin suspension (just visible turbidity) of a pure culture of *Azotobacter*, of which the same strain was used in all cases (the inoculation

principle of Christensen, 1915, as also applied by Ziemecka, 1932). The plaques were incubated at 28–30°C. and watched for macroscopic growth of *Azotobacter*, which, where present, became visible after 2 days and did not increase perceptibly after 4–5 days' incubation. Altogether 50 soils (46 wheat soils) were tested, in duplicate where sufficient soil was available. The results are seen in Table 12, where the amount of growth is indicated by the following characters:

0 : no visible growth of *Azotobacter*.
I : very thin growth of small confluent *Az.*-colonies.
II, III : amounts of growth intermediate between I and IV.
IV : maximal growth (thick slimy layer of *Azotobacter*).

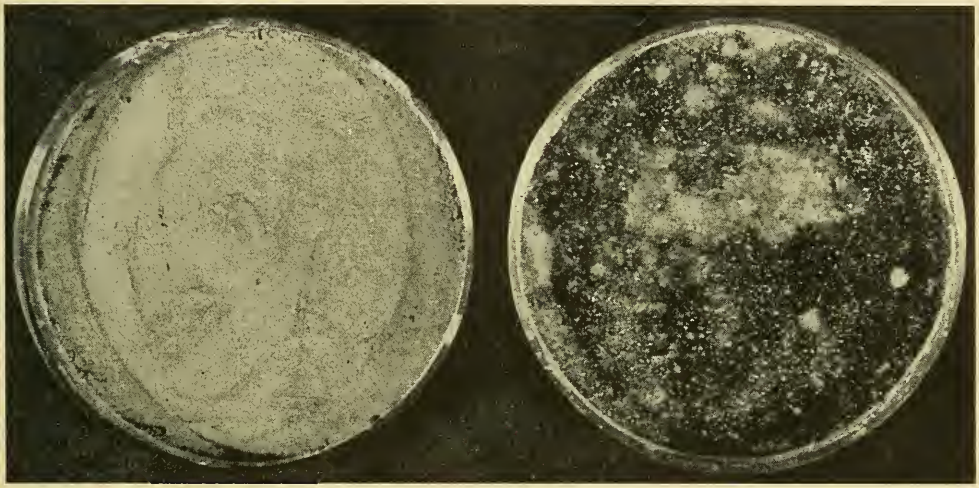

Fig. 5.—Development of *Azotobacter* on soil plaques. (Soil No. 29, inc. 5 d. 28-30°C.) Left: without phosphate (no visible growth of *Azotobacter*); right: with phosphate (maximal growth of *Azotobacter*). (⅔ nat. size.)

Figure 5 shows a good example of negative (0) and maximal (IV) development of *Azotobacter*. In some instances only a few isolated *Azotobacter*-colonies appeared instead of a confluent growth; in these cases the number of colonies is reported in the table. Without phosphate addition only one soil (No. 44, outside the wheat belt) gave a good growth, which was not further increased by phosphate addition, and only one wheat soil (No. 81) gave a fair growth without phosphate addition (incidentally, these two soils were among the richest in *Azotobacter*; cf. Table 2). Of all other soils, only a few gave a scanty growth without phosphate addition, and most of them none at all. When phosphate is added, the growth of *Azotobacter* becomes fair to excellent, except in a few cases (Nos. 11, 38, 65, and 82), where a stimulating effect of the phosphate is still often noticeable. This simple experiment thus shows that, even with addition of sufficient energy material, lime and molybdenum, practically all wheat soils here examined contain insufficient phosphate for a vigorous development of *Azotobacter*. It is interesting to observe that this applies also to highly productive soils like Nos. 26–29, 61, 62, 65, 66 and 68. This pronounced phosphate deficiency seems fully to explain why

TABLE 12.

Influence of Phosphate on Development of Azotobacter *on Soil Plaques.*

Soil No.	Growth of *Azotobacter.*	
	$-P_2O_5$.	$+P_2O_5$.
2	0	II
6	I	II
7	0	II
	0	II
11	0	(11 col.)
12	0	III
	0	III
13	0	III
	0	III
14	0	III
	0	III
15	0	0
22	0	II
25	0	II
26	0	IV
	0	IV
27	0	II
	0	II
28	0	II
	0	II
29	0	IV
	0	IV
31	0	III
	0	III
34	0	III
38	0	(7 col.)
42	0	III
	0	III
*44	III	III
45	0	II
	0	II
46	0	II
	0	II
47	0	III
	0	III
48	0	III
	0	III
51+52	(1 col.)	II
53+54	I	II
*55+56	0	II
58	0	III
*59	0	III
	0	III
*60	0	III
	0	III
61	0	IV
62	0	IV
63	0	IV
	(2 col.)	III
64	I	II
65	0	(1 col.)
	0	(36 col.)
66	0	III
67	0	II
68	(1 col.)	II
	(3 col.)	II
69	0	IV
74	0	III
	0	III
75	0	II
	0	II
76	0	II
	0	III
77	0	II
	0	II
78	0	II
	(3 col.)	II
79	0	II
	0	III
80	0	II
81	II	IV
82	0	(44 col.)
83	0	III
84	0	III
85	0	II

Soils marked * are outside the wheat belt.

approximately neutral to alkaline soils like Nos. 10, 11, 23, 24, and 30 failed to fix nitrogen on addition of glucose.

Nitrogen fixation experiments under anaerobic conditions were carried out on a small scale only. The first experiment was made on soil No. 21. Portions of 50 gm. air-dry soil were placed in small glass cylinders with glass lids, and received additions of 1·5% glucose, 1·0% $CaCO_3$, 0·004% Na_2HPO_4 (in solution), and 28% H_2O, which made the soil completely water-saturated. Two parallels were set up, and a glass slide was placed vertically in the middle of the soil column in each glass. The glasses were incubated at 28–30°C.; the slide in glass *a* was removed after 3 days and the one in glass *b* after 7 days, at which time the experiment was terminated, and microscopic counts and nitrogen determinations carried out. Gas formation was visible after 2–3 days. Plate counts after 7 days showed in both glasses a certain development of *Azotobacter*, which numbered 0·6–0·9 mill. per gm. (cf. Traaen, 1916). The next experiment was therefore carried out in the absence of oxygen. Two soils were used: (1) No. 30, with 1·5% glucose and 40% of a solution containing 0·05% Na_2HPO_4; and (2) No. 58 after previous incubation for 30 days and washing until free from nitrate (in order to have a soil of a low nitrogen content and free from mineral nitrogen); to this were added 1·5% glucose, 1·0% $CaCO_3$, and 21·4% of the same solution of Na_2HPO_4. The soil samples were placed in 100 c.c. glass beakers in a desiccator of about 1 litre capacity, where the oxygen was absorbed by means of pyrogallic acid and NaOH. The experiment was terminated after 7 days at 28–30°C.; gas formation and smell of butyric acid were also noticeable here. The results are seen in Table 13. (It should be pointed out that the initial nitrogen content of soil No. 58 is somewhat higher than should be expected after the washing out of the nitrate; this is due to the circumstance that the batch of calcium carbonate, although labelled "purissimum", was found to contain no less than 0·068% N; consequently the addition of 1·0% $CaCO_3$ adds 6·8 p.p.m. N to the soil.)

TABLE 13.

Nitrogen-fixation in Soils with 1·5% Glucose under Anaerobic Conditions.

Soil and Period of Incubation.	Total N, p.p.m., Mean ($\bar{x}$) (Catalyst : Se.).	n+1.	$S(x-\bar{x})^2$.	Gain of N, p.p.m., ($\bar{x}_2-\bar{x}_1$).	t.	P.
21. (+$CaCO_3$) Initial ..	582·8	9	352·0			
Inc. 7 d. (*a*).. ..	606·3	3	4·7	*23·5*	5·902	*<0·01*
(*b*) ..	592·0	4	204·0	9·2	2·153	0·1–0·05
58. (+$CaCO_3$) Initial ..	547·5	4	147·0			
Inc. 7 d.	608·0	6	766·0	*60·5*	8·774	*<0·01*
30. Initial..	1071·7	10	1334·1			
Inc. 7 d.	1124·0	4	298·0	*52·3*	7·580	*<0·01*

Direct counts :

No.	Total Bacteria. Mill. per gram.	Vegetative Clostridia. Mill. per gram.
21. Inc. 3 d. (*a*) ..	447±30·0	148±15·9
7 d. (*a*) ..	480±32·3	123±14·6
(*b*) ..	625±43·8	177±20·6
58. Inc. 7 d. ..	1276±74·0	663±47·1

In soil No. 21, which had been in contact with the air, the gain of nitrogen is significant only in glass *a*, and corresponds to a fixation of only 1·5 mgm. N per gm. glucose. In the other two soils the gains are higher, corresponding approximately to fixation of 3·5–4 mgm. N per gm. glucose, which is comparable to the yield in a vigorously fixing pure culture of *Clostridium pasteurianum*. The contact slides in soil No. 21 showed an abundance of big and typical clostridia, which stained particularly well after 3 days, but were more abundant, although largely Gram-negative, after 7 days. (See fig. 7, Pl. i.) The direct counts from soil No. 30 were lost by an accident; in the other two soils, organisms of the type of *Cl. pasteurianum* dominated the microscopical picture entirely. This is especially true of soil No. 58, where the fixation was stronger, and where the clostridia account for about one-half of the number of all the bacteria present; but if we consider their comparatively large cell-size, it becomes obvious that they account for much more than one-half of the total bulk of bacterial protoplasm present. Their variable and irregular shape and size make it very difficult to estimate their average cell-volume, but it is probably not far out to regard the average mature cell before sporulation as a cylinder of 1μ diameter and 5μ length, which corresponds to a volume of approximately 4μ^3. If we assume a content of 20% dry matter with 10% nitrogen, 1000 mill. cells will represent 0·08 mgm. N, and a calculation similar to the previous for *Azotobacter* gives:

Soil No.	Total Number of Clostridia, Mill. per gm.	Cell–N, p.p.m. (Calc.).	Total N Fixed, p.p.m.	Cell–N, % of Fixed N.
21 *a*, 3 d.	148	11·8	23·5	50
7 d.	123	9·8		42
21 *b*, 7 d.	177	14·2	(9·2)	—
58, 7 d.	663	53·0	60·5	88

These figures, although admittedly very roughly approximate, suggest that this process of fixation also consists in a simple synthesis of bacterial cells. If this is so, it seems unlikely that the process should be highly important under natural conditions in field soils, where vegetative clostridia are hardly ever present in numbers sufficient to be detected by microscopic methods.

We may now summarize the results of the experiments in this chapter:

Wheat soils with addition of glucose show, under aerobic conditions, only a very slight nitrogen-fixing capacity, and in most cases none at all, even if the soil reaction is favourable for the growth of *Azotobacter*. If nitrogen fixation takes place, it is accompanied by a development of *Azotobacter*, which does not multiply in soils of pH 6·0 and less, and may even fail to develop at higher pH-values, owing to phosphate deficiency. The process of fixation seems to consist in a simple synthesis of *Azotobacter*-cells, of which some 10 mill. per gm. of soil must be expected as required for the fixation of each p.p.m. of nitrogen. On the other hand, a multiplication of *Azotobacter* to a level incomparably higher than ever found under natural conditions (several millions per gm.) may take place without being accompanied by any measurable gain of nitrogen. The absolute lack of nitrogen fixation where no multiplication of *Azotobacter* has taken place proves conclusively that other types of organisms do not function as nitrogen-fixers in these soils under the conditions at hand. Nothing in the present results indicates

that special types of *Azotobacter*, such as those described by Altson (1936) and Starkey and De (1939), display any activity in acid soils.[22]

If nitrogen fixation is induced under anaerobic conditions, even the moderate gain of 3–4 mgm. of nitrogen per gm. of glucose is accompanied by a domination of the microscopic picture by vegetative clostridia.

Although these experimental results do not favour non-symbiotic nitrogen fixation as an important phenomenon in the wheat soils, we cannot, without reservation, generalize to apply them to field conditions, where such abnormally large concentrations of sugar are never present,[23] and where straw and root material of higher plants, especially wheat, represent the bulk of the organic matter added to the soil. The decomposition of these materials would evidently be accomplished by a microflora of a composition entirely different from that in the glucose experiments, and possibly including nitrogen fixing organisms unable to act in the presence of high concentrations of glucose. Also the behaviour of *Azotobacter* might under these conditions possibly be different from that in the glucose experiments. (An interesting experiment by Marr (1910) suggests that addition of straw to the soil may cause a much larger gain of nitrogen than addition of sugar.) We shall now see how the addition of such complex plant materials affects the nitrogen-fixing microorganisms and the nitrogen content of the soil.

3. *Soils with Addition of Straw, etc.*

Natural plant materials as sources of energy for nitrogen-fixing bacteria have been the subject of much research work with both pure and mixed bacterial cultures as well as with the soil itself.

The first experiments in this direction were due to Gerlach and Vogel (1902), who tried straw and green mustard as food substances for pure cultures of *Azotobacter*, with negative results. Dvorak (1912, cit. after Fulmer, 1917) found that several kinds of leaves, straw and clover contained substances that could be utilized for nitrogen-fixation by *Azotobacter*. Fulmer (1917) found only a slight nitrogen fixation by *Azotobacter* in solution with green clover and wheat, and Waksman and Hutchings (1937) found no fixation at all in sterile straw inoculated with *Azotobacter*. The results of such experiments must necessarily vary with the composition of the plant material, and they have only a limited significance, since most of the organic matter of (esp. mature) plants is represented by compounds that are not directly available to *Azotobacter* or *Clostridium pasteurianum*, but which may become so when partly broken down by other microorganisms. Among these compounds, celluloses and hemicelluloses occupy the first place; whether decomposition products from the lignins can serve as food

[22] The present author has not so far (Jan., 1940) been able to detect the presence of acid-resistant types of *Azotobacter* (*Az. indicum* Starkey and De) in Australian soils.

[23] Except perhaps where sugar or molasses has been added to the soil. Counts of *Azotobacter* in field soils under such treatment do not seem to have been carried out except in recent work in India by Dhar and Seshacharyulu (1936). The numbers of *Azotobacter* which they report in molasses-treated soil seem, however, quite fantastic—in certain instances 28,000 mill. per gm. of soil (by plate counting; the actual number of cells must have been at least a little higher). Assuming as before that 1000 mill. cells represent 0·1 mgm. N, this number of *Azotobacter*-cells alone would give the soil a nitrogen-content of 0·28%, whereas the actual *total* nitrogen-content was only about 0·1%! A misprint or miscalculation must be involved, unless, indeed, it be assumed that the *Azotobacter*-colonies originated from ultramicroscopic cell types; but the existence of these is still a matter for discussion.

material for nitrogen fixation is unknown, but does not appear likely, in view of the extreme slowness with which these compounds are decomposed.

Beijerinck (1904, cit. after Waksman, 1932) was the first to show that cellulose can serve for fixation of nitrogen in a crude culture of *Azotobacter* and anaerobic cellulose-decomposing bacteria. Pringsheim (1910) demonstrated the same phenomenon in mixed cultures of *Azotobacter* or *Cl. americanum* combined with anaerobic cellulose-decomposing bacteria;[24] the clostridia especially showed a much stronger nitrogen fixation than in ordinary pure cultures, in some cases no less than 12 mgm. N per gm. of cellulose decomposed. In combinations of *Azotobacter* and crude cultures of aerobic cellulose-decomposing bacteria, Hutchinson and Clayton (1919) found gains of up to 19 mgm. N per gm. of decomposed cellulose; this figure, indeed, may be too high; even the largest actual gain of nitrogen, about 5·6 mgm. per 50 c.c. of solution, could have taken place at the expense of the 0·5 gm. mannite also present; it may be that metabolic by-products of the cellulose-decomposing bacteria have stimulated *Azotobacter* to utilize the mannite more effectively than in the pure cultures, where the gains of nitrogen look remarkably low, or nitrogen may have been lost as ammonia from the pure *Azotobacter*-cultures during the long period of incubation (cf. Burk and Horner, 1936). Bucksteeg (1936) was unable to confirm these results with pure cultures of aerobic cellulose-decomposing bacteria, although crude cultures gave some fixation. Krishna (1928*b*) found only very small or negative gains of nitrogen in various combinations of nitrogen-fixing and cellulose-decomposing bacteria on both cellulose and straw (cf. Waksman and Hutchings, 1937). When cellulose is added to the soil and attacked by the natural soil microflora, its decomposition may result in large gains of nitrogen. Koch (1910) found that up to 10 mgm. N could be fixed per gm. of cellulose decomposed, but this depended largely on the nature of the cellulose-decomposing organisms. Engel (1931*b*) and Bucksteeg (1936) reported gains of 5–7 mgm. N per gm. of cellulose added to the soil, but did not determine the actual quantity of cellulose transformed. Engel found that a C/N ratio wider than 20/1 was necessary for nitrogen fixation. On the other hand, Vandecaveye and Villanueva (1934) found large gains of nitrogen in soils with addition of 1% filter paper to which had been added sufficient $NaNO_3$ to give it a content of 2·5% N; the C/N ratio of soil + filter paper and $NaNO_3$ was 10·2–13·6:1. The experiments are interesting because carbon determinations were also carried out, but the results look astonishing; for instance, in one case there is reported a gain of 532 p.p.m. nitrogen with a corresponding loss of 0·08%, or 800 p.p.m., carbon (Table 4, manured soil + lime and filter paper), i.e. 1 unit of N fixed per 1·5 units of C lost as carbon dioxide! This would be a nitrogen fixation of unparalleled economy, but one's confidence in the validity of the results becomes badly shaken by the circumstance that an almost equally high gain of nitrogen was reported in soil without any addition of organic matter.[25] It also appears surprising that such gains should have taken place although the numbers of *Azotobacter*, as determined by the silica-gel method, never exceeded 15,000 per

[24] It may be open to doubt whether methane fermentation of cellulose is actually due to pure cultures.

[25] Even more surprising is the circumstance that in one case (Virgin Palouse silt loam) in Vandecaveye and Villanueva's Table 4 the carbon content is actually stated to be *higher* after incubation than before, although the graph in Fig. 2 shows a vigorous CO_2-production. It is impossible to avoid the conclusion that some kind of sampling error must have influenced the results.

gm. The authors suggest the possible activity of organisms of the type studied by Greaves and co-workers (1928–32).

Hemicelluloses have not been studied much in this respect. Pringsheim and Pringsheim (1910) found that agar could be utilized by *Azotobacter* and *Cl. americanum* in combination with *Bact. gelaticum*. As in the experiments with cellulose, the clostridia displayed a remarkably great activity, and fixed up to 26 mgm. N per gm. of agar decomposed. It would seem that the nitrogen metabolism here is quite different from what happens in pure cultures of clostridia; Pringsheim's experiments, unfortunately, were few in number, and the whole problem urgently needs re-investigation. Koch (1909) found no gain of nitrogen in soil with addition of xylan. Diehm (1932) tried various hemicelluloses in soil experiments and found a sometimes very vigorous nitrogen fixation; the maximal gain corresponded to 31 mgm. N per gm. of galactan decomposed; the methods were not described in detail.

Straw and other natural plant materials have been studied much more extensively, especially in soil experiments. Koch et al. (1907) found no altogether conclusive evidence of nitrogen fixation in soil with addition of barley straw and no fixation, or loss, with filter paper, buckwheat or mustard hay. Remy (1909) found losses of nitrogen in soil with mustard plants or farmyard manure. Marr (1910) observed a large initial gain of nitrogen (corresponding to 8·8 mgm. per gm. of straw supplied) in soil with 0·5% straw, but this was followed by a loss of nitrogen on further incubation; addition of sugar gave rise to a much less intensive fixation. Tottingham (1916) and Richards (1917) observed nitrogen fixation in fermenting manures, even where *Azotobacter* did not appear to be present. Fulmer (1917) found only small gains of nitrogen in soils with green clover, wheat, or oats. Hutchinson (1918) found variable gains of nitrogen in soil with different kinds of leaves and straw; the maximum was about 6 mgm. N per gm. of straw in a sand-soil-mixture. Murray (1921) reported quite extraordinary gains of nitrogen,[26] even when ammonium sulphate was also added, and apparently independent of the quantity of straw added to the soil; similar gains were reported in soil without straw altogether, which makes the statement appear somewhat dubious. Meggitt (1923) found no gain of nitrogen on addition of straw to a soil that fixed large quantities when sugar was added. Zoond (1926) also obtained negative results with various plant materials. Engel (1931*a*) determined gains of nitrogen and losses of carbon in soil with different plant materials and manures. The gains were often considerable, occasionally as high as 1 part of nitrogen per 8 parts of carbon lost as CO_2 (sheep dung in soil after 47 days), but were followed by even bigger losses (cf. Marr, 1910). The analytical error was stated, and shows that the gains are fully significant (possibility of a sampling error?). Olsen (1932) studied nitrogen fixation in decomposing leaves of different trees; the largest gain was found in beech leaves, where it amounted to 180 mgm. N per 100 gm. original material, or approximately 10 mgm. per gm. of organic matter lost during the decomposition. Olsen made the important observation that more nitrogen was fixed under (partially) anaerobic than under fully aerobic conditions, and that the fixation stopped as soon as nitrate began to accumulate in the medium (cf. Burk and Lineweaver, 1930). Clostridia, but not *Azotobacter*, were found in the material. Desai (1933) reported

[26] For instance, 3·71 mgm. N per 10 gm. soil with 0·1% straw, or 12·95 mgm. with 0·4% straw, corresponding to 325-370 mgm. N per gm. of straw. It is interesting to compare these results with those of Vandecaveye and Villanueva (1934), as stated above; both refer to soils from Washington, U.S.A.

big gains of nitrogen in Indian soils with addition of maize straw, even 47·5 mgm. N per gm. of straw that had previously undergone fermentation. Makrinow (1935) observed a rich development of *Azotobacter* in decomposing straw, where microscopic counts showed up to about 1000 mill. per gm. dry matter, accompanied by a two- to three-fold increase in nitrogen content (it is not clearly seen how much of this is due to disappearance of organic matter as CO_2). Turk (1936) found fixation of up to 60 p.p.m. nitrogen in soils with 2% lucerne material, and De and Pain (1936) reported small gains of nitrogen in soils with rice straw; in both instances, gains of nitrogen were stated to take place in the same soils without addition of organic matter, which again makes these claims appear doubtful. Vandecaveye and Allen (1935) found no gain of nitrogen in soil with addition of straw, plus sufficient NH_4NO_3 to give the straw a content of 2·5% N, although gains were stated to take place in control soil without straw; this result disagrees strongly with that of the previous cellulose experiments (Vandecaveye and Villanueva, 1934).

One cannot help being impressed with the lack of agreement between all these experimental results, and in many cases also with the lack of precise information on experimental methods and errors of analysis, as discussed above. True evidence of intensive nitrogen fixation in arid soils is not supplied by these data.

One thing, however, stands out clearly: celluloses and hemicelluloses as well as natural plant materials *can* serve as energy material for nitrogen fixation if partially transformed by other microorganisms into compounds assimilable by the nitrogen-fixers, and this process of fixation can be very economical, since it may reach 10–25 mgm. N per gm. of transformed organic matter (Pringsheim, 1909–10; Koch, 1910; Olsen, 1932), of which a not negligible fraction must have been used up by the organisms that provide food for the nitrogen-fixers; this may even be the case where *Azotobacter* is not involved. Whether the process can take place with an even higher efficiency under soil conditions appears uncertain, and on the whole the quantitative extent of the process is very variable and depends on a number of incompletely-known factors; for instance, practically nothing is known about the nature and quantity of the decomposition products that serve as energy material for the nitrogen-fixers under soil conditions. There is here an important scope for future investigations.

A number of experiments have been carried out in order to ascertain whether a significant nitrogen fixation takes place in wheat soils during decomposition of straw and related materials.

Experiment No. 1: *Oats-straw in soil under aerobic conditions.*—Ten soils from Table 2 were tested, all of them except No. 8 being wheat soils; No. 8 was again used as a control soil with addition of 0·2% $CaHPO_4$. The air-dry soil samples received additions of 1·5% finely ground oats-straw, and distilled water to approximately 60% of their water-holding capacity. The arrangement of the experiment was otherwise the same as in the previous series (incubation without addition of organic matter, and experiments with glucose). After incubation for 30 days at 28–30°C., samples were taken for nitrogen determination, and the rest of the soil was incubated further: where two parallels were run, the residues of soil from the two dishes were combined into one sample after 30 days. A special arrangement was made with soil No. 30; the first analysis was not made until after 75 days, and the remaining soil was then divided into three portions, which were further incubated with addition of: (1) nothing, (2) 0·003% Na_2MoO_4, and

(3) 0·02% Na_2HPO_4. Also soil No. 7 was after 30 days divided into two portions, one of which was given an addition of 0·5% $CaCO_3$. Tests for *Azotobacter* were made during or after incubation, either by plate counting or in some of the first experiments by planting of straw particles on plates of silica-gel with mannite. No bacteria other than *Azotobacter* made any conspicuous growth on either agar or silica-gel; a few fungi, which produced a certain amount of growth, were tested for nitrogen-fixation with a negative result (cf. Table 4). Tests for nitrate and ammonia were also made; nitrate was always found to disappear completely after 30 days, but small quantities of ammonia (2–4 p.p.m. NH_3–N) were sometimes found at the termination of the experiment.

The results are found in Table 14, in which the initial nitrogen contents have been calculated on the content of nitrogen in 100 parts of soil (based on determinations before and after incubation without organic matter, as in Table 10) plus 1·5 parts of straw (which in air-dry condition contained 0·202% N), converted to 100 parts of soil-straw-mixture. The *t*-test for the significance of the changes in nitrogen content may without any significant error be based on the sum of squares of deviations ($S(x-\bar{x})^2$) referring to nitrogen determinations on soil alone, since the figure represented by the nitrogen determinations on the straw is negligible in comparison herewith (0·74 per 15 gm. of straw).

The analytical data in Table 14 show that there is in no instance any significant gain of nitrogen, even in soils No. 8 and 10, where *Azotobacter* has multiplied strongly during incubation. On the contrary, we find, as in the experiments with glucose, in several instances a definite loss of nitrogen, most apparent after prolonged incubation (cf. Marr, 1910, and Engel, 1931*a*), and which bears no relation to the presence or absence of *Azotobacter*. The cause of this loss is obscure; in some cases it may be due to denitrification, which can sometimes take place at moderate degrees of moisture (Lemmermann and Wichers, 1914), but this cannot apply in examples like Nos. 10 and 30, where the losses took place *after* the disappearance of all nitrate during the first period of incubation. It is noteworthy that the fertile alkaline soil No. 30, even with addition of molybdenum or phosphate, shows a loss of nitrogen as in the glucose experiments (Table 11).

According to this preliminary experiment, oats-straw seems quite unsuitable for nitrogen fixation, although in soils of favourable reaction it may stimulate the development of *Azotobacter* to an extent far surpassing the numbers ever found under natural soil conditions. This negative result was not due to this particular batch of straw being in itself unsuitable as a material for nitrogen fixation, as was shown by a preliminary control experiment started before the soil experiments: 20·0 gm. of straw plus 1 gm. of $CaCO_3$ were moistened with a 0·1% solution of K_2HPO_4, inoculated with a suspension of garden soil, and incubated in a moderately moist condition for 11 months at 28–30°C. After incubation the sample had lost approximately one-third (6·3 gm.) of its weight of dry matter and gained approximately 10% (11·0 mgm.) nitrogen, corresponding to a fixation of about 1·7 mgm. N per gm. of organic matter lost. *Azotobacter* developed vigorously, and numbered about 11 mill. per gm. of dry matter (by plate counting) at the end of the experiment. No nitrate and only traces of ammonia were found. In order to get more exact data on the value of straw as an energy material for nitrogen fixation, two experiments were now carried out in a medium poorer in nitrogen than natural soils.

TABLE 14.

Nitrogen-fixation in Soils with Addition of 1·5% Oats-Straw.

Soil No., Treatment and Time of Incubation.	Total N, p.p.m., Mean ($\bar{x}$).	n+1.	$S(x-\bar{x})^2$.	Gain (+) or Loss (−) of N, p.p.m., ($\bar{x}_2-\bar{x}_1$).	t.	P.
8. Initial..	467·0	11	804·0			
Inc. 30 d.	451·3	4	1118·8	*−15·7*	2·201	*0·05−0·02*
90 d.	468·2	5	1460·6	+1·2	0·175	0·9−0·8
Azotobacter: Initial, 9 per gm. 7 and 18 d., abundant on silica-gel. 43 d., 0·94 mill. per gm. 89 d., 1·87 mill. per gm.						
10. Initial..	652·4	10	1038·1			
Inc. 30 d. (*a*) ..	650·0	3	546·0	−2·4	0·304	0·8−0·7
(*b*) ..	652·0	3	74·0	−0·4	0·060	1·0−0·9
70 d.	634·3	3	58·7	*−18·1*	2·741	*0·02−0·01*
120 d.	624·0	5	534·0	*−28·4*	8·609	*<0·01*
Azotobacter: Initial, 20 per gm. 14 and 56 d., abundant on silica-gel. 107 d., 0·41 mill. per gm. Total count of bacteria: 1436±85·4 mill. *Azotobacter*-like: 8·9%.						
11. Initial..	654·6	14	2438·0			
Inc. 30 d. (*a*) ..	654·0	3	26·0	−0·6	0·074	1·0−0·9
(*b*) ..	643·5	4	961·0	−11·1	1·343	0·2−0·1
120 d.	655·0	5	826·0	+0·4	0·055	1·0−0·9
Azotobacter: Present initially. 14 and 42 d., fair on silica-gel. 120 d., 1700 per gm.						
14. Initial..	669·5	12	2361·5			
Inc. 30 d. (*a*) ..	641·0	3	474·0	*−28·5*	2·990	*0·02−0·01*
(*b*) ..	655·3	3	32·7	−14·2	1·621	0·2−0·1
75 d.	657·0	3	216·0	−12·5	1·375	0·2−0·1
Azotobacter: Present initially. 37 and 72 d., absent on dextrin-agar.						
15. Initial..	773·6	9	293·6			
Inc. 30 d. (*a*) ..	758·7	3	148·7	*−14·9*	2·812	*0·02−0·01*
(*b*) ..	760·3	3	484·7	−13·3	1·892	0·1−0·05
75 d.	754·5	4	323·0	*−19·1*	3·386	*<0·01*
Azotobacter: Absent initially and after 37 and 72 days.						
7. Initial..	751·4	11	4101·6			
Inc. 30 d. (*a*) ..	739·3	3	184·7	−12·1	0·983	0·4−0·3
(*b*) ..	717·3	3	88·7	*−34·1*	2·802	*0·02−0·01*
60 d.	731·7	3	188·7	−19·7	1·600	0·2−0·1
plus 0·5% $CaCO_3$ (added after 30 days)	742·8	4	524·8	−8·6	0·782	0·5−0·4
Azotobacter: Absent initially, after 30 days, and in soil +$CaCO_3$ after 40 days.						
1. Initial..	815·6	6	695·5			
Inc. 30 d.	812·0	3	6·0	−3·6	0·509	0·7−0·6
60 d.	832·1	3	418·7	+16·5	1·849	0·2−0·1
Azotobacter: Absent initially and after 59 days.						

TABLE 14.—*Continued.*

6. Initial..	844·7	11	2528·8			
Inc. 30 d. (*a*) ..	834·8	4	2038·6	−9·9	0·719	0·5−0·4
(*b*) ..	843·3	3	1922·7	−1·4	0·112	1·0−0·9
75 d.	811·9	4	1979·9	*−32·8*	3·016	*<0·01*
	Azotobacter: Absent initially and after 20 days.					
30. Initial..	1086·7	10	1034·1			
Inc. 75 d.	1096·2	5	1991·2	+9·5	1·137	0·3−0·2
140 d.	1066·7	3	848·7	*−20·0*	2·322	*0·05−0·02*
Do. +0·003% Na_2MoO_4*	1063·8	4	1734·6	*−22·9*	2·549	*0·05−0·02*
Do. +0·02% Na_2HPO_4*	1063·8	4	978·8	*−22·9*	2·989	*0·02−0·01*
	Azotobacter: Present initially. 12 and 75 d., absent. 140 d.: Control soil 1600, +Na_2MoO_4 1100, +Na_2HPO_4, 1040 per gm.					
2. Initial..	1269·1	6	2004·6			
Inc. 30 d.	1270·0	3	1112·0	+0·9	0·060	1·0−0·9
60 d.	1248·3	3	242·7	−20·8	1·642	0·2−0·1
	Azotobacter: Absent initially and after 59 days.					

* Salts added in solution after 75 days.

Experiment 2*a*: *Oats-straw in "synthetic" soil under aerobic conditions.*—A kind of artificial soil was made up from 80% sand, 18·5% pure kaolin, 1·0% $CaCO_3$, 0·3% Fe_2O_3, and 0·2% $CaHPO_4$. Portions of 500 gm. of this mixture plus 10 gm. oats-straw were placed in large crystallizing dishes (lower dish 18·5 cm. wide and 4·5 cm. deep), and moistened with 60 c.c. of a solution containing 0·1% $MgSO_4$, 0·1% KCl, 0·05% NaCl, 0·05% $FeCl_3$, and 0·001% Na_2MoO_4; moreover, 5 c.c. of a 1:10 suspension of a soil rich in *Azotobacter* (No. 9, Table 2) were added as an inoculum to each dish. The soil-straw-mixture was in this way moistened to approximately 60% of its water-holding capacity. The dishes were incubated at 28–30°C., and nitrogen was determined at the start and periodically during 181 days; at intervals, plate counts of *Azotobacter* and direct counts of total bacteria were also carried out. The experiment was started in duplicate, but after 120 days the contents of the two dishes were combined into one. After one week numerous tiny brown glistening drops of mucus could be seen on the surface of the medium by means of a hand lens; microscopic examination showed these drops to be colonies of *Azotobacter*, practically free from other bacteria. The results are given in Table 15.

Both the plate counts and the direct counts show a luxuriant growth of *Azotobacter*, which is represented by numbers comparable with those in the glucose experiments. The numbers reach their maximum after 30–60 days, and then fall only slowly. The direct microscopic counts of *Azotobacter*-like organisms are only 2 to 5 times as high as the plate counts, and all through the experimental period they account for some 20–25% of the total bacterial numbers. If we consider the actual size of the cells, the *Azotobacter* account for a much larger proportion of the total mass of bacterial protoplasm; the rest of the population consisted chiefly of the small rods and cocci seen in normal soils, which hardly have an average volume of more than $1\mu^3$, or about one-fifth of the volume of a normal *Azotobacter*-cell. Compared with this both absolutely and relatively

TABLE 15.

Nitrogen-fixation in Sand-kaolin Mixture with 2·0% Oats-Straw.

Time of Incubation.	Total N, p.p.m., Mean ($\bar{x}$).	n+1.	$S(x-\bar{x})^2$.	Gain of N, p.p.m. ($\bar{x}_2-\bar{x}_1$).	t.	P.
Initial	66·1	4	195·5			
60 d. (*a*)..	85·5	3	11·76	*+19·4*	3·954	*0·02—0·01*
(*b*)	86·1	3	8·66	*+20·0*	4·097	*<0·01*
120 d. (*a*)..	84·4	3	8·78	*+18·3*	3·748	*0·02—0·01*
(*b*)	79·8	3	27·13	*+13·7*	2·688	*0·05—0·02*
181 d. (*a*+*b*)	78·8	3	3·05	*+12·7*	2·639	*0·05—0·02*
Addition of 0·1% glucose after 181 d. inc. 4 days.						
Initial	78·8	3	3·05			
4 d. (*a*)	94·3	3	0·42	*+15·5*	20·35	*<0·01*
(*b*)	94·0	3	1·65	*+15·2*	17·17	*<0·01*

Number of Microorganisms:

Time of Incubation.	Direct Counts.			Plate Counts of *Azotobacter*, Mill. per gm.
	Total Bacteria. Mill. per gm.	*Azotobacter*-like.		
		% of Total.	Mill. per gm.	
10 d. (*a*)..	1262±75·7	16·9	213±25	
(*b*)	904±66·6	22·0	199±28	
15 d. (*a*)..	894±56·4	20·9	187±23	44·9
(*b*)	905±60·4	20·4	185±24	48·3
30 d. (*a*)..	930±59·4	24·6	229±31	70·8
(*b*)	750±56·7	23·9	179±25	67·6
60 d. (*a*)..	701±50·5	24·8	174±23	69·9
(*b*)	734±53·4	16·7	123±19	64·5
120 d. (*a*)..	640±40·2	14·2	91±13	51·4
(*b*)	480±31·3	10·4	50±9	32·8
181 d. (*a*+*b*)	273±23·2	13·9	38±9	21·9

abundant development of *Azotobacter*, the gain of nitrogen is only very moderate; after 60 days it amounts to about 20 p.p.m., and by further incubation it not only does not increase, but actually declines a little, as in some of the experiments in soil (Table 14). If, as before, we assume that 1000 mill. *Azotobacter*-cells represent 0·1 mgm. cell-nitrogen, we find after 60 days 12 and 17 p.p.m. nitrogen represented by the 123 and 174 mill. cells per gm. by direct counting, or 60 and 85% of all the nitrogen fixed. When it is remembered that large numbers of cells must have arisen and died before this time, it seems clear that the gain of nitrogen is due to simple synthesis of cell material. After this time it seems that the cells have continued to exist in a resting condition without fixing more nitrogen.

When the experiment was terminated, the medium contained no nitrate and only a minute quantity (1·0 p.p.m.) of NH_4–N. A control experiment was now carried out: a portion of the air-dry material was re-moistened to its original

moisture content, given an extra addition of 0·1% glucose, and incubated for 4 days at 28–30°C. Total nitrogen was then determined. This addition of a small quantity of available energy material resulted in a vigorous nitrogen fixation, corresponding to 15–16 mgm. N per gm. of glucose, and similar to the yield in a vigorously fixing pure culture. This shows plainly that the inactivity of *Azotobacter* after the 60-day period was not due to any active inhibitory factor, but presumably to a failing supply of available organic nutrients.

While this experiment proves that straw can serve as energy material for nitrogen fixation under conditions favourable for the growth of *Azotobacter*, it also shows that the gain is comparatively small and only corresponds to about 1·0 mgm. N per gm. of straw present—a quantity which would indeed have been difficult to detect in a soil of normal nitrogen content with 1·5% straw; also the small quantities of nitrate and ammonia normally present in the soil would automatically tend to counteract this slight fixation.

As shown by Olsen (1932), nitrogen fixation on the basis of complex plant materials may be more vigorous under partially anaerobic[27] than under fully aerobic conditions. An experiment to test this point was therefore carried out with the straw.

Experiment 2*b*: *Oats-straw in water-saturated medium.*—For this experiment a pure sand medium was used, with addition of 1·0% $CaCO_3$ and 0·2% $CaHPO_4$. Portions of 800 gm. sand plus 12 gm. straw were moistened with 195 c.c. nutrient solution (0·1% $MgSO_4$, 0·1% KCl, 0·1% $FeCl_3$, 0·001% Na_2MoO_4) and 5 c.c. of the same soil suspension as the previous experiment. This saturated the sand-straw-mixture completely, without forming an actual layer of liquid over the surface. Duplicate dishes, as in Experiment 2*a*, were incubated at 28–30°C., and after 100 days combined into one sample. The results of this experiment are seen in Table 16.

The development of *Azotobacter* is here only moderate in comparison with the previous experiment, although this organism is constantly present, in numbers of 1 to 3 mill. per gm. in the period from 28–60 days, during which time the nitrogen fixation was most intense. The direct microscopy certainly showed presence of *Azotobacter*-like cells, but they were too few to admit of any reliable counting, especially in the later periods. The nitrogen fixation is far more vigorous than under aerobic conditions; during the first 4 weeks it is only moderate, but during the next 32 days approximately 30 p.p.m. nitrogen are fixed; the rate of fixation then becomes slower, and at the end of the experiment the total gain (93 p.p.m.) corresponds to a fixation of approximately 6 mgm. N per gm. of straw originally supplied—a figure very similar to the maximal fixation found by Hutchinson (1918) with straw in soil-sand mixture. This stronger fixation might be due, as Olsen (1932) suggests, to a production of larger amounts of organic by-products suitable as nutrients for the nitrogen-fixers. Whether *Azotobacter* or other organisms were chiefly responsible for the fixation is a more difficult question. There can be little doubt, however, that the activity of *Azotobacter* may account for the gains during the first two months of the experiment, although their numbers were much lower than in the previous experiment under aerobic conditions. It is very likely that the higher water-content would favour the development of protozoa (numerous active ciliates and flagellates could actually

[27] The "anaerobic" conditions in Olsen's experiments were produced merely by supersaturation of the leaf material with water. Aerobic organisms could thus still have displayed some activity on the surface of the medium.

TABLE 16.

Nitrogen-fixation in Water-saturated Sand with 1·5% Oats-straw.

Time of Incubation.	Total N, p.p.m., Mean ($\bar{x}$).	n+1.	$S(x-\bar{x})^2$.	Gain of N, p.p.m. ($\bar{x}_2-\bar{x}_1$).	t.	P.	*Azotobacter* Plate Count. Millions per gm.
Initial ..	50·5	3	3·71				
28 d. (*a*)	67·3	3	0·67	*16·8*	19·66	*<0·01*	3·05
(*b*)	60·0	3	0·86	*9·5*	10·79	*<0·01*	2·85
60 d. (*a*)..	95·9	3	0·05	*45·4*	*	—	1·03
(*b*)	88·9	3	19·23	*38·4*	—	—	2·24
100 d. (*a*)..	112·3	3	2·11	*61·8*	—	—	0·56
(*b*)	100·1	3	2·09	*49·6*	—	—	0·55
116 d. (*a*+*b*)					—	—	0·41
150 d. ..	123·6	4	8·84	*73·1*	—	—	0·047
179 d. ..					—	—	0·012
200 d. ..	138·6	4	5·03	*88·1*	—	—	0·010
250 d. ..	143·8	3	4·03	*93·3*	—	—	0·006

Direct counts, millions per gram:

Time.	Total Bacteria.	*Azotobacter*-like Org., % of Total.	
28 d. (*a*)..	310±25·1	8·5	
(*b*)	480±46·3	5·9	
60 d. (*a*)..	932±44·7	4·7	Typical clostridia were seen only sporadically
(*b*)	910±48·5	2·1	(less than 0·1% of total). Numerous short,
100 d. (*a*)..	764±36·7	2·0	plump, vibrio-like bacteria, and some long,
(*b*)	638±36·4	1·7	slender rods with oval terminal spores,
150 d. ..	1485±72·7	1·4	closely resembling anaerobic cellulose-
200 d. ..	591±35·5	0·5	decomposing bacteria.

* In this and subsequent cases the values of *t* are much higher than after 28 days, and the calculation therefore superfluous.

be seen by direct microscopic examination of the fluid from the sand), which by feeding on the *Azotobacter*-cells would increase their death-rate and thereby prevent them from accumulating as resting cells in such numbers as observed under aerobic conditions, while still permitting the renewed production of young and active *Azotobacter*-cells, as suggested by Cutler and Bal (1926). The fixation of 45 p.p.m. nitrogen in the first 60 days would require a production of 450 mill. cells per gm., or an average daily production of 7·5 mill. new cells per gm. (assuming, as before, that 1000 mill. cells equal 0·1 mgm. N)—a figure not incompatible with the numbers of 1 to 3 mill. *Azotobacter* found by plate counting. The actual numbers, although they could not be counted with any accuracy, must certainly have been somewhat higher; it must be remembered that we know nothing about the rates of reproduction and death of *Azotobacter*-cells in the soil, but there is nothing obviously wrong in the assumption that a stationary population of some 2–3 mill. *Azotobacter* could have renewed itself 3 times during 24 hours, thereby producing a total of 6–9 mill. new cells per gm., which in the present case may safely be assumed to have taken all their nitrogen from the atmosphere, since no trace of nitrate or ammonia could ever be detected in the medium. (It should not be forgotten, however, that the growth of *Azotobacter* must have taken place in the surface layers of the medium; in the depth the conditions were

completely anaerobic, as shown by the clearly visible formation of iron sulphide.) During the last 100 days of the experiment this explanation seems definitely to break down. The gain of nitrogen in this period amounts to 20 p.p.m., which would require a total production of some 200 mill. *Azotobacter*-cells, or an average daily formation of 2 mill. new cells per gm.—a rate of reproduction which is difficult to reconcile with the relatively low numbers (6,000 to 47,000) shown by the counts; and even these numbers fall steadily throughout the period. Unless we make the assumption (for which there is no positive evidence) that the relation between nitrogen fixation and cell production in *Azotobacter* has here been quite different from that in pure cultures, where the fixation is "growth-bound", we cannot escape the conclusion that in this experiment other organisms must, at least in the later stages, have contributed to the fixation. It does not appear very likely that these other organisms should be *Cl. pasteurianum* and related types, since vegetative clostridia were hardly ever visible in the direct counts, in contrast to the experiments with glucose (Table 12), where they dominated the picture, while carrying out only a moderate fixation. But in view of the much longer duration of the present experiments, no very definite conclusion can be drawn. It may also be that we have here a case of "non-specific" nitrogen fixation due to other organisms producing nascent hydrogen in their metabolism; this possibility is worthy of further investigation (cf. Clausen's (1931) remarkable findings on nitrogen fixation by anaerobic cellulose-decomposing bacteria).

While all these considerations should not be taken for more than an attempt to explain the mechanism of nitrogen fixation, the two experiments have shown conclusively that straw can indeed serve as energy material for an appreciable fixation of nitrogen, but more so under semi-anaerobic than under aerobic conditions—a result in sharp contrast to the frequently expressed views concerning a particularly intensive nitrogen fixation under arid soil conditions, but in perfect agreement with the findings of Olsen (1932); indeed, the difference is much more pronounced here than in Olsen's experiments with leaf material.[28] We shall now see what happens in natural soil under conditions of high moisture and addition of straw.

Experiment 3*a*: *Oats-straw in water-saturated soil.*—This was carried out with a typical wheat soil of moderately acid reaction, with and without addition of calcium carbonate. The soil was a mixture of equal parts of soils No. 14 and 15 (Table 2), which had been washed free from nitrate and then air-dried. Duplicate portions of 250 gm. air-dry soil were given additions of 1·0% ground oats-straw, ± 1·0% $CaCO_3$, and 0·005% Na_2HPO_4, and were moistened with 46 c.c. H_2O plus 1 c.c. of a 1:10 suspension of soil No. 21 after incubation anaerobically with glucose (Table 12); this inoculum served to introduce both *Azotobacter* and *Cl. pasteurianum*. The addition of moisture was sufficient to saturate the soil completely, so that it formed a compact plaque, about 12 mm. deep, in the usual big Petri dishes used for incubation of soils. The samples were incubated at 28–30°C. for 120 days, and periodical nitrogen determinations, direct counts, and plate counts of *Azotobacter* were carried out; after 78 days the two parallel dishes were combined into one. After 4 days' incubation the dishes with addition of $CaCO_3$ showed on their surface numerous small drop-like bacterial colonies which after 6–7 days turned dark and microscopically revealed themselves as *Azotobacter*-

[28] In this connection it is also interesting to note that Richards (1917) found nitrogen fixation in dung with extra addition of water, but not in material of normal moisture content.

colonies; the picture was similar to that of the soil plaques with addition of starch. When the soils had been disturbed after the first sampling (14 days) this growth did not reappear. The dishes without $CaCO_3$ remained permanently free from macroscopically visible growth of *Azotobacter*, but showed quite a conspicuous growth of black fungal mycelium on the surface. The experimental data are seen in Table 17.

TABLE 17.

Nitrogen-fixation in Water-saturated Soil (Mixture of Nos. 14 and 15) plus 1·0% Oats-straw.

Time of Incubation.	Total N, p.p.m., Mean ($\bar{x}$). (Catalyst: Se).	n+1.	$S(x-\bar{x})^2$.	Gain (+) or Loss (−) of N, p.p.m., ($\bar{x}_2-\bar{x}_1$).	t	P.	*Azotobacter* Plate Count. 1,000 per gram.
I. $-CaCO_3$. pH initial: 5·6. pH final: 6·0.							
Initial ..	680·3	4	224·76				0·190
70 d. (*a*)..	688·5	2	14·05	+8·2	1·225	0·3−0·2	
(*b*)	681·7	3	10·67	+1·4	0·267	0·8−0·7	
90 d. (*a*+*b*)							1·40
120 d. (*a*+*b*)	684·3	4	5·56	+4·0	0·913	0·4−0·3	4·40
II. +1·0% $CaCO_3$. pH final: 7·7.							
Initial ..	681·3	4	104·76				0·190
14 d. (*a*)..	666·7	3	98·67	*−14·6*	2·997	*0·05−0·02*	1320·0
(*b*)	680·0	3	96·00	*−1·3*	0·269	0·8−0·7	1780·0
70 d. (*a*)..	699·7	3	180·67	*+18·4*	3·189	*0·05−0·02*	1174·0
(*b*)	692·3	3	4·67	*+11·0*	3·078	*0·05−0·02*	2075·0
90 d. (*a*+*b*)							3370·0
120 d. (*a*+*b*)	694·0	4	54·00	*+12·7*	3·490	*0·02−0·01*	3840·0

Direct Counts.	Total Bacteria, Mill. per gm.	*Azotobacter*-like Organisms. % of Total.	*Azotobacter*-like Organisms. Mill. per gm.	
$-CaCO_3$, 70 d. (*a*) .	753±62·9	1·0	(8)	
(*b*) ..	421±40·7	2·3	(10)	Vegetative clostridia were
$+CaCO_3$, 70 d. (*a*) ..	548±49·3	0·7	(4)	seen sporadically only
(*b*)	668±58·7	2·8	(19)	(less than 1% of total).
120 d. ..	636±50·8	3·8	(22)	

In soil without lime the reaction becomes slightly less acid during incubation, but it still remains unfavourable for the growth of *Azotobacter*. In spite of this, *Azotobacter* has multiplied to a certain extent after 90–120 days, when it is present in numbers rarely found under natural soil conditions, even at favourable reaction. About two-thirds of the colonies counted after 120 days were *Az. vinelandii*—the only occasion when this species was encountered.[29] Although a strain isolated herefrom showed vigorous nitrogen-fixation in pure culture

[29] The colonies were rather smaller and less compact than those of *Az. chroococcum*, and remained pure white on dextrine agar. On agar with glucose, saccharose or mannite the organism grew better and produced the typical greenish-yellow soluble pigment. Morphologically the organism was similar to *Az. chroococcum*, but showed an active motility in young cultures.

(Table 4), the soil shows no gain of nitrogen that even begins to approach significance. In the dishes with addition of lime the macroscopic growth of *Azotobacter* is reflected in the very large numbers found by plate counting after 14 days and for the rest of the experimental period, during which they tend to rise rather than to fall. The nitrogen-figures show no gain during the first 14 days, when the first explosive development of *Azotobacter* took place; actually one of the dishes shows a loss which appears significant. After 70 days the gain becomes only just significant, and remains so after 120 days, when it corresponds to a fixation of about 1·3 mgm. N per gm. of straw added to the soil. The final gain of 12·7 p.p.m. nitrogen would only require a total production of some 130 mill. *Azotobacter*-cells per gm. of soil, a figure that might well be expected where the plate counts show numbers of about 1 to 4 mill. per gm. The direct counts towards the end of the experiment are relatively low and not much influenced by the lime. The *Azotobacter*-like organisms are too few to admit of any reliable counting, and the extremely sparse representation of clostridia (which never showed a typical morphological appearance) does not suggest that they had taken any part in the fixation. It may here be mentioned that other observations on soil plaques of the same depth and water saturation as used in this experiment, but with addition of glucose, showed a vigorous growth of clostridia;[30] their absence in this experiment was thus not due to a too high oxygen-tension.

Experiment 3*b*: *Oats-straw in water-saturated soil.*—This was a smaller experiment, carried out in connection with the previous one, on soils No. 10 and 11 after incubation for 30 days with addition of 1·5% straw, which did not result in any fixation (Exp. 1, Table 13). Equal parts of samples *a* and *b* (75 gm. of No. 10, 40 gm. of No. 11) were moistened to complete saturation and incubated at 28–30°C. in small glass cylinders with lids that did not fit air-tightly. Nitrogen was determined in No. 10 after 62 and 90 days, in No. 11 only after 35 days; at the same time plate counts of *Azotobacter* were carried out. Table 18 gives the results.

TABLE 18.

Nitrogen-fixation in Water-saturated Soils with 1·5% Oats-straw.

Soil No. and Time of Incubation.	Total No. p.p.m., Mean ($\bar{x}$).	n+1.	$S(x-\bar{x})^2$.	Gain (+) or Loss (−) of N, p.p.m. ($\bar{x}_2-\bar{x}_1$).	t.	P.	*Azotobacter* 1,000 per gm.
10. Initial ..	651·0	6	626·0	—	—	—	—
Inc. 62 d.	667·3	3	28·7	*+16·3*	2·442	*0·05—0·02*	321·0
90 d.	654·7	3	88·7	+3·7	0·518	0·7—0·6	630·0
11. Initial ..	648·0	7	1176·0	—	—	—	0·54
Inc. 35 d.	637·0	3	122·0	−11·0	1·249	0·3—0·2	4·70

Soil No. 10 shows, after 62 days, a barely significant gain of nitrogen, corresponding to about 1·1 mgm. N per gm. of straw originally introduced, but after 90 days this has become non-significant, although the numbers of *Azotobacter* have increased. Small quantities of nitrate and ammonia could be detected at this

[30] The starch plaques (Table 12) also frequently showed gas bubbles and smell of butyric acid.

stage. In soil No. 11 the multiplication of *Azotobacter* is only slight, and no gain of nitrogen has taken place; no nitrate or ammonia was present.

These experiments show that even a long-protracted decomposition of oats-straw in water-saturated soil will even at the best (soil with addition of lime and phosphate) result in only a very moderate fixation of nitrogen not exceeding 2 mgm. N per gm. of straw introduced, and this is associated with a vigorous multiplication of *Azotobacter*. Another experiment was now set up with wheat straw, which represents a more important source of organic matter in the wheat soils.

Experiment 4: *Wheat straw in soil of high and low moisture.*—A faintly acid soil (pH 6·3) of very low humus content (equal parts of soils No. 31 and 33, Table 2) was used. Portions of 500 gm. air-dry soil received additions of 5·0 gm. finely ground wheat straw, 50 c.c. of a solution containing 0·15% K_2HPO_4 and 0·015% Na_2MoO_4, and 10 c.c. of a 1:20 suspension of soil No. 44 to ensure the presence of *Azotobacter*. The soil portions were placed in 10 cm. wide glass jars covered with Petri dishes, and were given the following extra additions: Jars 1–2: Nothing; Jars 3–4: 5·0 gm. $CaCO_3$; Jars 5–6: 30·0 c.c. H_2O; Jars 7–8: 30·0 c.c. H_2O + 5·0 gm. $CaCO_3$.

The extra addition of water made the soil completely saturated. The jars were incubated at 28–30°C., and nitrogen-determinations and plate counts of *Azotobacter* were carried out on the original material as well as periodically during 3 months of incubation. (The initial nitrogen-determinations on soil with and without $CaCO_3$ agreed within the analytical error, which was rather large in the former case; the average was therefore taken to represent the initial nitrogen content.) In the jars with high moisture, and particularly in those with lime (Nos. 7–8), growth of *Azotobacter* became visible as large slimy colonies on the surface after 3–4 days (Pl. i, fig. 6). The rest of the data are seen in Table 19.

TABLE 19.

Nitrogen-fixation in Soil of High and Low Moisture Content, with addition of Wheat-straw.

Treatment and Time of Analysis.	Total N, p.p.m., Mean ($\bar{x}$). (Catalyst : Se.)	n+1.	$S(x-\bar{x})^2$.	Gain (+) or Loss (−) of N, p.p.m. ($\bar{x}_2-\bar{x}_1$).	t.	P.	*Azotobacter* 1,000 per gm.
Initial :							
Soil−$CaCO_3$	223·4	4	14·94	—	—	—	
Soil+$CaCO_3$	227·1	6	215·45	—	—	—	
Average ..	225·6	10	265·44	—	—	—	0·018
Soil−$CaCO_3$, low moisture (10·8% H_2O).							
7 d. (*a*) ..	—	—	—	—	—	—	(>100)
(*b*) ..	—	—	—	—	—	—	(>100)
14 d. (*a*) ..	—	—	—	—	—	—	862
(*b*) ..	—	—	—	—	—	—	964
28 d. (*a*) ..	227·1	3	20·18	+1·5	0·447	0·7−0·6	1,240
(*b*) ..	226·3	2	6·48	+0·7	0·248	0·9−0·8	934
56 d. (*a*) ..	225·2	3	6·72	−0·4	0·122	1·0−0·9	460
(*b*) ..	218·4	3	2·66	−7·2	2·216	*0·05−0·02*	345
90 d. (*a*) ..	221·1	3	5·82	−4·5	1·375	0·2−0·1	176
(*b*) ..	220·7	3	21·25	−4·9	1·442	0·2−0·1	60

TABLE 19—*Continued.*

Soil+$CaCO_3$, low moisture (10·8% H_2O).

7 d. (*a*)	..	—	—	—	—	—	—	1,500
(*b*)	..	—	—	—	—	—	—	1,830
14 d. (*a*)	..	—	—	—	—	—	—	5,820
(*b*)	..	—	—	—	—	—	—	5,380
28 d. (*a*)	..	227·1	3	11·65	+1·5	0·454	0·7–0·6	2,711
(*b*)	..	223·1	3	1·46	−2·5	0·771	0·5–0·4	2,018
56 d. (*a*)	..	225·7	3	6·58	+0·1	0·031	1·0–0·9	1,710
(*b*)	..	225·9	3	46·89	+0·3	0·086	1·0–0·9	846
90 d. (*a*)	..	221·7	3	11·53	−3·9	1·180	0·3–0·2	1,599
(*b*)	..	221·7	3	11·53	−3·9	1·180	0·3–0·2	868

Soil−$CaCO_3$. high moisture (15·4% H_2O).

7 d. (*a*)	..	—	—	—	—	—	—	1,017
(*b*)	..	—	—	—	—	—	—	1,658
14 d. (*a*)	..	—	—	—	—	—	—	1,563
(*b*)	..	—	—	—	—	—	—	3,625
28 d. (*a*)	..	233·2	3	0·33	*+7·6*	2·351	*0·05–0·02*	1,840
(*b*)	..	236·2	3	2·81	*+10·6*	3·261	*<0·01*	6,540
56 d. (*a*)	..	233·0	3	2·47	*+7·4*	2·272	*0·05–0·02*	3,390
(*b*)	..	243·1	3	11·76	*+17·5*	5·296	*<0·01*	5,330
90 d. (*a*)	..	236·8	3	0·89	*+11·2*	3·458	*<0·01*	5,697
(*b*)	..	251·8	3	7·39	*+26·2*	7·850	*<0·01*	9,444

Soil+$CaCO_3$, high moisture (15·4% H_2O).

7 d. (*a*)	..	—	—	—	—	—	—	5,650
(*b*)	..	—	—	—	—	—	—	4,880
14 d. (*a*)	..	—	—	—	—	—	—	7,470
(*b*)	..	—	—	—	—	—	—	6,540
28 d. (*a*)	..	245·1	3	13·81	*+19·5*	5·879	*<0·01*	9,504
(*b*)	..	242·1	3	6·80	*+16·5*	5·038	*<0·01*	10,170
56 d. (*a*)	..	244·4	3	14·64	*+18·8*	5·660	*<0·01*	7,340
(*b*)	..	239·6	2	2·64	*+14·0*	3·491	*<0·01*	7,130
90 d. (*a*)	..	258·1	3	0·99	*+32·5*	10·03	*<0·01*	4,420
(*b*)	..	252·9	3	7·05	*+27·3*	8·333	*<0·01*	5,414

Soil+$CaCO_3$, low moisture, after 90 d., with extra addition of 0·5% Ca-acetate.

Initial	..	221·7	6	23·06	—	—		—
7 d.	..	240·7	3	6·59	*+19·0*	13·06	*<0·01*	11,3500

pH of soil initially: 6·3. pH values after 90 days:

−$CaCO_3$, low moisture	(*a*) 6·1	+$CaCO_3$, low moisture	(*a*) 7·8
	(*b*) 6·2		(*b*) 7·8
high moisture	(*a*) 6·8	high moisture	(*a*) 7·9
	(*b*) 6·9		(*b*) 8·0

The soil of low moisture-content shows, both with and without addition of lime, a very strong multiplication of *Azotobacter*, which reaches its maximum after 2–4 weeks and then recedes somewhat; this is most pronounced in the soil without lime, where the numbers also, as might be expected, are considerably

lower than where lime is added. In both series, however, there is absolutely no indication of any gain of nitrogen; on the contrary, a small loss of nitrogen seems to have taken place in one of the jars in the series without lime after 56 days. A very different picture is seen at high degree of moisture. The numbers of *Azotobacter* are considerably higher than in the previous series; in soil with lime they reach a maximum of about 10 mill. per gm. after 4 weeks and then fall only slowly, but in the soil without lime they continue to rise steadily throughout the whole period; this phenomenon is reflected in the circumstance that the originally faintly acid soil in this series had become almost neutral at the end of the experiment, as shown at the bottom of the table (cf. Olsen (1932) on beech and oak leaves under anaerobic conditions). The nitrogen-figures show significant but somewhat irregular gains of nitrogen. In the series with lime the increase is rapid at first, seems to stop in the period from 38 to 56 days, and then begins again. In the unlimed soil the gain of nitrogen takes place less rapidly, but in one of the jars it reaches the same level as in the soil with lime after 90 days; the disagreement between these two parallel jars corresponds to a similar difference in the numbers of *Azotobacter*, which are consistently higher in jar *b*, where the stronger fixation has taken place.

These results are quite in agreement with those found in the previous experiments with oats-straw: absolutely no nitrogen fixation under aerobic conditions, even where the conditions for the growth of *Azotobacter* are optimal, and where these organisms actually multiply to the extent of several millions per gm. of soil—and under semi-anaerobic conditions after incubation for 3 months only a moderately strong fixation, corresponding to 2·5–3·0 mgm. N per gm. of straw originally added. This gain is apparently due to *Azotobacter*, which here flourishes even more strongly than under fully aerobic conditions. This last phenomenon is rather surprising in view of the essentially aerobic character of *Azotobacter*; the best explanation would seem to be that under semi-anaerobic conditions larger quantities of organic matter become available as carbonaceous food for *Azotobacter*, through the decomposition of the straw cellulose and hemicelluloses, which are not directly available to *Azotobacter*. Now it is well known that fungi, which play a very important part in the decomposition of cellulose in soil under aerobic conditions, transform the cellulose almost quantitatively into mycelial substance, carbon dioxide, and water (Waksman, 1932), and in pure cultures of most aerobic cellulose-decomposing bacteria there is little or no accumulation of soluble organic compounds, unless growth is suspended by high temperature or by exclusion of the oxygen and the cellulose-decomposing enzymes thus enabled to act independently (Kalnins, 1930). On the other hand, anaerobic cellulose-decomposing bacteria produce large quantities of organic acids which are excellent sources of energy for *Azotobacter* (Waksman (1932) quotes data showing 50–66% of the cellulose being converted into fatty acids by the organisms of Omeliansky, and 75% of the cellulose-carbon into acetic acid and ethyl alcohol by the *Clostridium thermocellum* Viljoen et al.), and the same thing may apply to the decomposition of hemicelluloses. That the lack of such decomposition products is the reason why no fixation took place under aerobic conditions was strongly suggested by a control experiment with soil from the third series (low moisture, addition of $CaCO_3$) of Experiment 4 after incubation for 90 days. A mixture of equal parts of soil from jars *a* and *b* was given an addition of 0·5% calcium acetate and 12% H_2O, and incubated for 7 days at 28–30°C.; nitrogen determination and plate count of *Azotobacter* were then carried out. The data

are included in Table 19. As in the similar experiment with addition of glucose to sand-kaolin-mixture in Experiment 2*a*, the provision of a small quantity of directly available energy material immediately resulted in a vigorous nitrogen fixation, which corresponds to very nearly 5 mgm. N per gm. of acetic acid, and which is accompanied by a luxuriant growth of *Azotobacter*; the production of approximately 110 mill. *Azotobacter*-cells per gm. of soil shown by the plate count would alone, provided the cells were of normal average size, account for a fixation of 11 p.p.m. N, or more than half the quantity actually fixed.

Another experiment shows the importance of a sufficient supply of soluble decomposition products even more strikingly.

Experiment No. 5: *Filter paper in soil under aerobic conditions.*—This experiment was originally planned in the hope of obtaining quantitative data on the correlation between numbers of *Azotobacter*, fixation of nitrogen, and decomposition of cellulose. The soil used here was similar to that of the previous experiment—a light sand soil very poor in humus and of faintly acid reaction (No. 32, Table 2). Duplicate portions of 830 gm. air-dry soil received additions of 1·0% finely ground filter paper, 1·0% $CaCO_3$, and 12·5% of a nutrient solution containing 0·4% K_2HPO_4 and 0·04% Na_2MoO_4. Incubation took place in the same large glass dishes as in Experiment No. 2. A special inoculum was prepared in order to ensure the presence of both nitrogen-fixing and actively cellulose-decomposing bacteria able to exist in symbiosis with each other: 3 gm. of strips of filter paper and 2 gm. of $CaCO_3$ were covered with 40 c.c. of a nitrogen-free nutrient solution in a deep Petri dish, infected with soil No. 44, and incubated at 28–30°C. After a few weeks the paper appeared visibly decayed, and colonies of *Azotobacter* started to grow on the pieces of paper that reached the surface; an emulsion of this material was used as inoculum, at the rate of 0·5 c.c. per 100 gm. soil. The results are seen in Table 20.

The paper showed no visible attack by microorganisms, and quantitative determination of the cellulose was not continued. The numbers of *Azotobacter* declined steadily during two months of incubation and no significant gain of nitrogen took place. The experiment was discontinued after 60 days, and control experiments were set up with duplicate portions of 60 gm. air-dry soil re-moistened to their previous degree of moisture and with further addition of: (*a*) 0·2% glucose; (*b*) 0·5% calcium lactate; (*c*) 0·5% calcium acetate.

The samples with glucose and lactate were incubated for 7 days and those with acetate for 8 days at 28–30°C., when nitrogen was determined and direct and plate counts of *Azotobacter* carried out. As shown in Table 20, these additions resulted in a vigorous nitrogen fixation, which corresponds to very nearly 14 mgm. N per gm. of glucose and 5·3–6·5 mgm. per gm. of salt of the two organic acids (or 7·3–8·2 mgm. per gm. of acid). As in the control experiments with glucose and Ca-acetate belonging to Experiments No. 2*a* and 4, this is comparable with the yield in a vigorously fixing pure culture of *Azotobacter*; particularly the acetate seems here to have been utilized very efficiently (Mockeridge (1915) and Gainey (1928) found only 2·6–3·8 mgm. N fixed by *Azotobacter*, per gm. of acetic acid consumed, whereas Hunter (1923) briefly mentions a fixation of 8 mgm. N per gm. of potassium acetate; it should be noted that no molybdenum was added to the media in any of these experiments). The fixation was bound up with an enormous development of *Azotobacter*, which, especially in the soil with lactate, rose to surprising numbers. The microscopic counts showed that the numbers of *Azotobacter*-like organisms were only about

3 times as high as the plate counts (as in Exp. 2*a* in this series, and the glucose-experiments in Table 11 where the plate counts were high). The *Azotobacter*-like cells in this experiment appeared rather small, and this was particularly the case in soil plus lactate (cf. Winogradsky, 1926), where they were little more than 1μ in diameter, but intensely staining (Pl. i, fig. 5). Since the actual size of the living cells cannot be ascertained, we cannot calculate the amount of cell nitrogen with any reasonable accuracy.

TABLE 20.

Nitrogen-fixation in Sand Soil with Cellulose, and with Low Concentrations of Glucose or Salts of Organic Acids.

Time of Incubation.	Total N, p.p.m., Mean ($\bar{x}$). (Catalyst: Se.)	n+1.	$S(x-\bar{x})^2$.	Gain of N, p.p.m. ($\bar{x}_2-\bar{x}_1$).	t.	P.	*Azotobacter* Plate Count Millions per gm.
I. Soil No. 32, with 1% $CaCO_3$ and 1% filter paper.							
Initial ..	246·0	4	58·0				0·127
18 d. (*a*) ..							0·081
30 d. (*a*) ..	249·0	3	26·0	3·0	0·958	0·4—0·3	0·067
(*b*) ..	249·7	3	34·67	3·7	1·125	0·4—0·3	0·071
60 d. (*a*) ..	252·3	3	18·67	6·3	1·916	0·2—0·1	0·041
(*b*) ..	248·7	3	2·67	2·7	1·015	0·4—0·3	0·036
II. After 60 days, addition of (*a*) 0·2% glucose, (*b*) 0·5% Ca-lactate, (*c*) 0·5% Ca-acetate.							
Initial ..	250·5	6	41·5				
Glucose:							
Inc. 7 d. (*a*)	279·0	3	38·0	*28·5*	11·96	*<0·01*	78·1
(*b*)	278·3	3	33·67	*27·8*	12·00	*<0·01*	75·9
Lactate:							
7 d. (*a*) ..	283·3	3	24·67	*32·8*	15·09	*<0·01*	214·0
(*b*) ..	277·3	3	24·67	*26·8*	12·33	*<0·01*	232·0
Acetate:							
8 d. (*a*) ..	277·0	3	6·00	*26·5*	15·06	*<0·01*	91·2
(*b*) ..	277·0	3	8·00	*26·5*	14·09	*<0·01*	81·6

Direct Counts.	Total Bacteria. Mill. per gm.	*Azotobacter*-like Org. Mill. per gm.	Average Diameter of *Azotobacter*-like Cells (μ).
Glucose 7 d. (*a*) ..	1133±71·4	212±26·2	1·29
(*b*) ..	958±58·1	199±22·5	
Lactate 7 d. (*a*) ..	1615±87·9	603±44·8	1·04
(*b*) ..	1488±76·6	566±39·9	
Acetate 8 d. (*a*) ..	893±57·1	250±27·3	1·11
(*b*) ..	750±53·3	237±26·5	

This experiment shows plainly that even where the general soil conditions for the growth of *Azotobacter* are excellent (temperature, reaction, aeration, supply of phosphate and molybdenum), and where we have supplied a potential source of energy (cellulose) and organisms capable of converting this into food material for *Azotobacter* in solution cultures, there may still not be a sufficient production of available organic matter to induce a measurable fixation of nitrogen (cf. Koch, 1910); it seems that the required cellulose-decomposing bacteria do not find

suitable conditions for development in moderately moist soil, or at least fail to produce soluble organic compounds from the cellulose (cf. the important observation of Kalnins (1930) that aerobic cellulose-decomposing bacteria produce more reducing sugars when deprived of oxygen). In Experiments No. 2–4 there was always, except in the acid soil in Exp. 3*a*, a rapid multiplication of *Azotobacter* after the addition of straw, which thus seems to contain certain substances directly available to *Azotobacter* (cf. Dvorak, cit. after Fulmer (1917) and Waksman (1932)). According to Waksman and Hutchings (1937), *Azotobacter* can utilize the water-soluble constituents of oats-straw without, however, fixing any nitrogen, and Hunter (1923) found nitrogen fixation in glucose solution slightly stimulated by addition of straw. Some experiments were carried out in order to ascertain the value of the water-soluble constituents as well as the insoluble residue of straw as energy materials for nitrogen fixation.

Experiment No. 6: *Water-extract of wheat straw as a medium for* Azotobacter.—60 gm. of finely-ground wheat straw were heated for 1 hour in the steamer with 600 c.c. H_2O, filtered, and washed on the filter with distilled water until the filtrate had been made up to 600 c.c. The extracted straw was further washed repeatedly with distilled water and then dried. The extract was given an addition of 0·02% K_2HPO_4, 0·01% $FeCl_3$, 0·005% Na_2MoO_4, and 0·4% $CaCO_3$, distributed in portions of 50 c.c. in 300 c.c. Erlenmeyer flasks, and sterilized. Before the addition of the salts the extract was found to contain 0·125% dry matter with 9% ash, i.e. 50 c.c. of medium contains 0·570 gm. dry organic matter. The flasks were inoculated with *Az. vinelandii* and *Az. chroococcum* (strain 34), and cultures as well as controls incubated at 28–30°C. The results are given in Table 21.

TABLE 21.

Nitrogen-fixation by Azotobacter *in Water-extract of Wheat-straw.*

Culture.	Incubation Days.	Total N per Culture, Mgm.	Gain of Nitrogen, Mgm.	
			Per Culture.	Per gm. of Org. Matter Present.
Control initially (*a*)	0	2·60 } 2·66	—	—
(*b*)	0	2·72 }		
Control incubated (*a*)	8	2·13	—	—
(*b*)	8	2·48	—	—
Az. vinelandii (*a*)	7	6·22	3·56	6·2
(*b*)	7	6·20	3·54	6·2
Az. chroococcum (*a*)	8	4·48	1·82	3·2
(*b*)	8	4·74	2·08	3·6
(*c*)	14	7·88	5·22	9·2

The sterile control medium appeared to lose a small quantity of nitrogen during incubation (cf. M. Löhnis, 1930); the initial nitrogen content has therefore been subtracted from that of the cultures. Both strains of *Azotobacter* made a good and rapid growth; *Az. vinelandii* fixed about 3·5 mgm. N after 7 days, and *Az. chroococcum* even more than 5 mgm. after 14 days. These gains corresponded to about 6 to 9 mgm. N per gm. of total organic matter present, but this was probably not all used up, and the utilization must be regarded as quite economical,

since it compares well with the utilization of glucose (Table 4).[31] This shows plainly that some of the water-soluble constituents of the straw are directly available to *Azotobacter* and may serve for the fixation of a certain amount of nitrogen (unlike the result obtained by Waksman and Hutchings, 1937). But since 50 c.c. of medium represent the soluble constituents of 5 gm. straw, even the fixation of 5·22 mgm. N corresponds to little more than 1 mgm. N per gm. of straw (incidentally, this is very similar to the gain in Exp. 2*a* with oats-straw), and under soil conditions this may easily become nullified if only small quantities of nitrate or ammonia are present and become utilized by *Azotobacter* in preference to atmospheric nitrogen; it must also be remembered that the presence of ammonia and nitrate will enable non-nitrogen-fixing organisms to compete with *Azotobacter* for the available energy material. The soil in Experiment 4 contained initially 6·2 p.p.m. NH_4– + NO_3–N, and the soils in Experiment 1 even more (see Table 28). In addition to this, some more mineral nitrogen is undoubtedly produced and re-assimilated during incubation. This makes it quite understandable that, if only the water-soluble constituents of the straw are available, *Azotobacter* may flourish abundantly in the soil without fixing nitrogen. In the artificial soil in

TABLE 22.

Multiplication of Azotobacter *and Fixation of Nitrogen in Synthetic Soil with Addition of Untreated and Water-extracted Wheat Straw.*

Treatment.	Time of Incubation.	*Azotobacter*, Thousands per gram.	
		Low Moisture.	High Moisture.
Untreated straw.	Initial	0·019	0·019
	8 days	13430·0	25740·0
	15	20088·0	57090·0
	21	23650·0	86150·0
Water-extracted straw.	Initial	0·019	0·019
	7 days	14·6	61·7
	14	24·0	36·6
	21	7·5	524·0
	28	4·8	140·0

Nitrogen-fixation (Catalyst: Se).

Treatment.	Total N., p.p.m., Mean ($\bar{x}$).	n+1.	$S(x-\bar{x})^2$.	Gain of N., p.p.m. ($\bar{x}_2-\bar{x}_1$).	, t.	P.
Untreated, initial ..	55·9	4	13·74	—	—	—
Inc. 21 d., low moisture	66·9	3	14·25	*+11·0*	6·087	*<0·01*
Inc. 21 d., high moisture	98·0	2	8·82	*+42·1*	20·47	*<0·01*
H_2O-extracted, initial ..	42·9	4	1·38	—	—	—
Inc. 28 d., low moisture	49·0	3	2·99	*+6·1*	8·553	*<0·01*
Inc. 28 d., high moisture	51·4	3	3·98	*+8·5*	10·75	*<0·01*

[31] Some of the organic matter in the straw extract consisted of reducing sugars; another part is probably represented by gums, which are also directly available to *Azotobacter*, as shown by Mockeridge (1915).

Experiment 2*a*, on the other hand, no mineral nitrogen was present, and *Azotobacter* only had recourse to the atmospheric nitrogen, thereby causing the small gain observed.

Experiment No. 7: *Untreated and water-extracted wheat straw in "synthetic" soil.*—An artificial soil similar to that in Experiment 2*a* was made up from 85% pure sand, 14% kaolin, and 1% $CaCO_3$; to this were added 2% untreated or water-extracted straw, and 12·5% of a nutrient solution containing 0·1% K_2HPO_4, 0·05% $MgSO_4$, 0·02% $FeCl_3$, 0·01% Na_2MoO_4; the solution also included a suspension of soil from Experiment 4 (low moisture, + $CaCO_3$). Portions of 90 gm. were placed in Petri dishes, two of which were given an extra addition of 7·5% H_2O; this saturated the medium completely. Incubation at 28–30°C. with periodical plate counts of *Azotobacter* and nitrogen determination after 3 and 4 weeks gave the results shown in Table 22.

The dishes with untreated straw show an abundant development of *Azotobacter*, especially at high moisture, where the colonies became macroscopically visible on the surface. Small quantities of nitrogen are fixed; the amounts correspond to approximately 0·6 mgm. N per gm. of straw at low and 2·1 mgm. at high moisture, and are roughly proportional to the final numbers of *Azotobacter*. The theoretical numbers of *Azotobacter*-cells of normal size required to bring about this amount of fixation would be 110 mill. per gm. at low and 420 mill. per gm. at high moisture, of which thus the final plate counts account for 20–25%.

The removal of the water-soluble constituents from the straw has reduced the development of *Azotobacter* enormously, especially at low moisture, where their numbers are not much higher than may be found in natural soil. In both cases there is an apparently significant but very small gain of nitrogen (corresponding to 0·3–0·4 mgm. per gm. of straw added) which, if real, could hardly be explained as simple synthesis of *Azotobacter*-cells, especially at low moisture. The plates showed an abundant growth of excessively polysaccharide-forming bacteria similar to the "bacille gommeux" of Winogradsky (1926), which might conceivably have fixed some nitrogen here; but since the experiment was not carried out in duplicate and on rather small quantities of material, no great significance can be attached to it. Another experiment was therefore made with water-extracted straw under aerobic conditions, with larger quantities of material and a longer period of incubation. The possible influence of soil organic matter was also tested by addition of 10% of a wheat soil rich in humus (No. 81, Table 2).

Experiment No. 8: *Water-extracted wheat straw in "synthetic" ± natural soil.*—The basal synthetic soil was the same as in the previous experiment; portions of 600 gm. were given the following additions:

(*a*) 1·5% extracted straw
(*b*) Do. + 10% soil No. 81
(*c*) 10% soil alone
} + 12·5% nutrient solution and inoculum as in Exp. 7.

Incubation took place in large crystallizing dishes for 8 weeks at 28–30°C. The results are given in Table 23.

In the first series (straw alone) there is a moderate development of *Azotobacter*, as in the previous experiment, but absolutely no gain of nitrogen, which renders the significance of the previous experiment doubtful. The extra addition of humus-rich soil (ser. *b*) has reduced the development of *Azotobacter*, which hardly shows a significant multiplication, and no significant gain of

TABLE 23.

Multiplication of Azotobacter *and Fixation of Nitrogen in Synthetic Soil ± Natural Soil, with Addition of Water-extracted Wheat Straw.*

I. Multiplication of *Azotobacter*.

Time of Incubation.	Plate Counts of *Azotobacter*, 1000 per gm. of Soil.		
	Series (*a*). (Straw Alone.)	Series (*b*). (Straw+Soil.)	Series (*c*). (Soil Alone.)
Initial	0·028	0·194	0·127
8 days	39·7	(<0·7)*	0·15
28 days	18·0	0·28	0·13
42 days	14·2	0·33	0·13
56 days	12·1	0·32	0·19

II. Nitrogen content (Catalyst: Se).

Treatment and Time.	Total N, Mean p.p.m. ($\bar{x}$).	n+1.	$S(x-\bar{x})^2$.	Gain (+) or Loss (−) of N, p.p.m. ($\bar{x}_2-\bar{x}_1$).	t.	P.
(*a*) (straw):						
Initial	36·8	3	7·61	—	—	—
28 days..	37·5	3	2·69	+0·7	0·517	0·7−0·6
56 days..	36·0	2	0·13	−0·8	0·544	0·7−0·6
(*b*) (straw+soil):						
Initial	175·9	3	3·44	—	—	—
28 days..	178·6	3	18·17	+2·7	1·423	0·3−0·2
56 days..	177·7	3	36·35	+1·8	0·683	0·6−0·5
(*c*) (soil):						
Initial	157·6	4	85·62	—	—	—
28 days..	158·7	3	25·85	+1·1	0·305	0·8−0·7
56 days..	153·8	4	1·10	−3·8	1·414	0·3−0·2

* Too high dilution.

nitrogen has taken place. This effect can hardly be ascribed to any other cause than the provision of available nitrogen by the soil, which has enabled other organisms to suppress *Azotobacter*. The medium contained initially a mere trace of nitrate and ammonia, but in the third series (addition of soil alone) there was, after 28 days, a content of 11·9 p.p.m. NO_3–N, and after 56 days of 10·9 p.p.m. In this series the numbers of *Azotobacter* have remained practically stationary, and the nitrogen content has not changed significantly (cf. Table 7). It has been claimed by Bortels (1936) that sodium molybdate in a concentration of 0·001% will enable *Azotobacter* to assimilate free nitrogen in the presence of nitrate, although this is also largely utilized. The present findings are of some interest in showing that even a higher concentration of Na_2MoO_4 (0·01%) does not necessarily help *Azotobacter* from being suppressed by other organisms in the presence of available nitrogen and shortage of available organic food material, such as in ser. *b*.

The series of experiments, Nos. 1–8, now justifies the following conclusions:

Under aerobic soil conditions only the water-soluble constituents of oats- and wheat-straw become available to *Azotobacter*, and only in artificial soil free from mineral nitrogen do we observe a moderate fixation of nitrogen, which does not materially exceed 1 mgm. N per gm. of straw present. No fixation has ever been found to take place in the absence of *Azotobacter*, and has been found with certainty only in cases where *Azotobacter* has reached numbers incomparably higher (millions per gm.) than under natural soil conditions; thus there is nothing to suggest that organisms of other groups will utilize the organic matter of the straw for nitrogen fixation. The fact that vigorous nitrogen fixation and strong development of *Azotobacter* may be induced by extra addition of small quantities of sugar or salts of organic acids indicates that organisms capable of producing such compounds from the insoluble constituents of the straw do not develop under fully aerobic soil conditions.

When semi-anaerobic conditions are created by saturation of the soil with water, a stronger fixation of nitrogen may be induced, but this is a slow process, since 8 months at 28–30°C. were required for fixation of 6 mgm. N per gm. of oats straw in sand, and 3 months for fixation of 3 mgm. N per gm. of wheat straw in soil.

From these results it is obvious that the straw residues of wheat and oats crops do not represent a very valuable form of energy material for nitrogen fixation under the conditions normally existing in Australian wheat soils.

Besides the residues of straw and stubble, we must reckon with the organic matter of the plant roots as a potential energy material, which will always be left behind by the crops, while the greater part of the straw will usually be destroyed by stubble-burning or carried away as hay.

The value of root material of cereals for nitrogen fixation does not seem yet to have been investigated. An experiment in this direction was therefore carried out. Roots of wheat plants (from flowering to ripening stage) that had served for *Azotobacter*-counts in the rhizosphere (Table 3) were collected, dried at 98°C., and ground finely. A synthetic soil was made up from 90% pure sand, 9% kaolin, and 1% $CaCO_3$; to this were added 2·5% root material (containing 3·51% H_2O and 36·8% organic matter as loss on ignition, thus introducing 0·92% organic matter into the medium), 10% of a nutrient solution containing 0·25% K_2HPO_4, 0·1% $MgSO_4$, 0·1% $FeCl_3$, and 0·005% Na_2MoO_4, besides 1% suspension 1:5 of soil No. 44 as inoculum. Portions of 250 gm. of medium were placed in 4 glass jars similar to those used in Experiment 4, and two of the jars were given an extra addition of 8% H_2O to give complete water-saturation. They were then incubated at 28–30°C., and periodical nitrogen determinations and *Azotobacter*-counts carried out. The results are shown in Table 24.

At low moisture content there is only a moderately strong development of *Azotobacter*, but after 60 days there is quite a significant gain of nitrogen, corresponding to approximately 1·5 mgm. N per gm. of organic matter introduced with the root material. It might seem that in this case some nitrogen may have been fixed by organisms other than *Azotobacter*: the numbers of this organism seem rather too low to account for the fixation of 13·7 p.p.m. nitrogen; theoretically some 140 mill. cells of normal size would have to be produced, i.e., there should have been an average daily production of 2 mill. new cells per gm. of medium, which is about 30 to 80 times as high as the numbers observed by plate counting; but since no direct counts were carried out in this series, the question

TABLE 24.

Nitrogen-fixation in Sand-kaolin Mixture with Wheat Root Material.

Time of Incubation.	Total N, p.p.m., Mean ($\bar{x}$). Catalyst: Se.	n+1.	$S(x-\bar{x})^2$.	Gain (+) or Loss (−) of N, p.p.m. ($x_2-\bar{x}_1$).	t.	P.	*Azotobacter*, Plate Count. 1,000 per gm.
I. Aerobic conditions (10·6% H_2O).							
Initial ..	91·1	4	20·01				0·024
14 d. (*a*) ..							24·3
30 d. (*a*) ..	94·7	3	3·98	+3·6	2·152	0·1−0·05	65·2
(*b*) ..	96·3	3	57·68	+5·2	1·731	0·2−0·1	32·0
60 d. (*a*+*b*)	104·8	4	8·08	*+13·7*	8·954	*<0·01*	24·2
II. Semi-anaerobic conditions (17·1% H_2O).							
Initial ..	91·1	4	20·01				0·024
14 d. (*a*) ..							6050·0
30 d. (*a*) ..	103·2	3	5·53	*+12·1*	6·978	*<0·01*	3340·0
(*b*) ..	105·2	3	5·89	*+14·1*	8·111	*<0·01*	6660·0
60 d. (*a*+*b*)	110·6	4	5·06	*+19·5*	13·49	*<0·01*	2220·0
120 d. (*a*+*b*)	115·6	3	23·15	*+24·5*	10·92	*<0·01*	319·0

Direct counts in material under semi-anaerobic conditions:

	Total Bacteria, Mill. per gm.	*Azotobacter*-like Org., % of Total.	
30 d. (*a*)	555±42·7	5·0	Vegetative clostridia were observed only sporadically, about 0·5–1·5% of total.
(*b*)	497±38·3	6·0	
60 d.	394±29·9	5·5	

cannot be answered with certainty. A small quantity of nitrate (0·4 p.p.m. NO_3–N) was present after 60 days; it is therefore unlikely that the fixation would have proceeded beyond this stage.

Where semi-anaerobic conditions have been created by full water-saturation of the medium, we find the same effect as in the experiments with straw: *Azotobacter* develops abundantly during the first 30 days and then declines, especially in the second half of the experimental period. Nitrogen fixation is comparatively vigorous during the first 30 days, where the numbers of *Azotobacter* are highest, and then becomes slower; during the last 60 days, when the numbers of *Azotobacter* show a marked drop, the fixation amounts to only 5 p.p.m. or 20% of the total fixation. The total yield of fixed nitrogen, 24·5 p.p.m. (which corresponds to approximately 2·7 mgm. per gm. of organic matter), would theoretically require a total production of 245 mill. normal *Azotobacter*-cells per gm., or an average of

about 2 mill. new cells daily, which might well be expected in comparison with the figures shown by plate counting. The direct counts of total bacteria are comparatively low, and clostridia form only a very insignificant proportion hereof. Everything thus suggests that the fixation is entirely due to *Azotobacter*.

The general result of this experiment thus leads to the conclusion that the organic root material is not much different from the straw as a source of energy for nitrogen fixation; if only a moderate degree of fixation shall be obtained (2·5–3 mgm. N per gm. of organic matter supplied), a high degree of moisture must be maintained for a period of several months.

If we now would try to form an estimate of the quantities of nitrogen that could be fixed on the basis of the residues of straw, stubble and roots of the cereal crops (mainly wheat), we must consider (1) the amounts of organic matter represented by these residues, and (2) the soil conditions under which the decomposition of this organic matter takes place.

The amount of wheat straw may roughly be estimated at 1½ times the weight of the grain, or approximately 100 lb. straw per bushel of wheat. The weight of the roots is less easy to assess; according to Harris (1914) and numerous earlier data collected by Miller (1916), it may rise to 25% of the weight of the top portions of the plants at low yield and low soil moisture. Richardson (1923), on the other hand, found the weight of the wheat roots amounting to only 3 to 11% of the total plant. We can therefore not reckon with more than, at the most, 20% of the weight of the tops, or about 30 lb. (= 20% of 60 lb. grain plus 100 lb. straw) root material per bushel. The total 130 lb. of straw and root material will not all be returned to the soil, since in ordinary farming practice most of the straw is either burned or eaten by sheep if the stubble fields are grazed before fallowing; it is unlikely that more than one-third of the straw will be left as stubble. Generally speaking, we may say that there will be at the most some 60 to 80 lb. of root and stubble residue per bushel of wheat grain available as a potential energy material for nitrogen fixation. (This corresponds to some 700 to 1000 lb. residue per acre from an average 12-bushel wheat crop; oaten or wheaten hay crops may similarly be expected to leave some 400–500 lb. of residue per ton of hay produced).

To these amounts of residue we should correctly add the organic matter which the soil receives during the growth of the crops in the form of organic root secretions and decayed root particles. The quantity of these can only be guessed, but is hardly considerable. Lyon and Wilson (1924) found that maize plants in aseptic water culture liberated organic compounds in quantities equal to 1–3% of the dry weight of the plants; they considered it probable (although this does not seem obvious) that the yield would be higher under natural conditions. It will probably be a liberal estimate to assume that the wheat plants may give off altogether 5% of their total dry weight as root secretions plus root tissue during the growth period. This would correspond to somewhat less than 10 lb. per bushel of grain produced (= 5% of 60 lb. grain plus 100 lb. straw plus 30 lb. roots). We may safely disregard the importance of these small quantities of organic matter as energy material for nitrogen fixation, in view of the apparent unsuitability of the actual root secretions as food material for *Azotobacter*, as shown by Krasilnikov (1934), and the present observations (Table 3) which show that even under favourable soil conditions *Azotobacter* is less stimulated in the neighbourhood of the roots than is the general soil microflora.

As to the soil conditions, we may first of all safely conclude that no nitrogen will be fixed during the decomposition of the residues in the very numerous soils where acid reaction or phosphate deficiency prevents *Azotobacter* from multiplying when organic matter is made available. But even where reaction and phosphate supply are optimal, it may be difficult to have all other conditions fulfilled, especially moisture and temperature; besides, there is the disturbing influence of nitrate and ammonia in the soil,[32] which must always be expected to counteract the nitrogen fixation, if not to suppress it altogether. A degree of moisture in the surface soil, approaching saturation and persisting for longer periods (which we must consider necessary for an effective utilization of the residues), can only be expected in periods of high rainfall and low rate of evaporation, i.e. the months of June–August in districts with winter maximum of rainfall; but this is associated with a low soil temperature. Systematic observations on soil temperatures in the wheat districts of New South Wales have not yet been made. Observations in South Australia by Prescott and Piper (1930) and in Victoria by Richardson (1923) and Penman and Rountree (1932) have shown mean soil temperatures of 6·5 to 10·5°C. at a depth of 6 inches during these months, when the moisture content of the surface soil is highest. Such temperatures are definitely unfavourable for nitrogen fixation, both in pure cultures of *Azotobacter* (Burk, 1934)[33] and in the soil itself (Koch et al., 1907; Hutchinson, 1918). Higher moisture content in the subsoil may indeed exist under more favourable temperature conditions, but here there is no significant supply of organic matter from the plant residues. The nitrate concentration is lowest in soil under crops in their later stages of growth and for some time after harvesting (Prescott and Piper, 1930; Penman and Rountree, 1932), but during the growth of the crop there are only the small quantities of root material to act as energy material for nitrogen fixation, and during the later stages of growth (September–November) no high degree of soil moisture can be expected. This is even more true of the period after harvesting, when the soil is either left as stubble land or subjected to short fallowing—also of the northern parts of the wheat belt in New South Wales, where short fallowing is most common and most of the rain falls in the summer months, but the rate of evaporation is high owing to the summer temperature.

Thus we can neither in summer time (when crop residues are ploughed into the soil immediately after harvest and before short fallowing) nor in winter time (when the residues are ploughed under in the autumn before long fallowing) expect really optimal conditions for nitrogen fixation on the basis of these residues. The same applies to the stubble fields, where only the roots are actually incorporated into the soil, and where the degree of moisture is usually not high except in wet autumns. On stubble fields there is often a considerable growth of weeds, self-sown wheat, etc., parts of which will serve to augment the amount of actual crop residues when the stubble field is ploughed in the autumn; the

[32] The moisture and temperature conditions in wheat soils have been discussed by Richardson (1923), Richardson and Fricke (1931), Prescott and Piper (1930), and Penman and Rountree (1932). Data on nitrate content at different seasons have been given by Taylor (1922), Prescott and Piper (1930), and Penman and Rountree (1932). Cf. also Prescott (1934).

[33] A number of strains of *Azotobacter* from the present experiments were tested for growth at 9-11°C. Only one strain (No. 79) produced a fair but very slow growth at this temperature, and another (No. 44) a mere trace of growth. No significant activity of *Azotobacter* can therefore be expected in seasons where such soil temperatures prevail.

quantity of this material is very variable and difficult to estimate, especially as much of it is usually eaten off by sheep. Since at least some of it represents a younger type of plant material than the cereal straw and roots, it might be expected to be more easily decomposible and therefore more readily available for nitrogen fixation, but against this we must set the higher nitrogen content of young plant material (Waksman, 1932) with consequent tendency to ammonia and nitrate production; under ordinary circumstances this material can hardly be considered of much significance, especially since its decomposition will take place first during the winter months, where the moisture conditions may indeed be favourable for nitrogen fixation but under unfavourable temperature conditions, and afterwards in spring time with lower moisture and increasing nitrate production.

Even under exceptionally favourable combinations of moisture, temperature, soil reaction, supply of mineral nutrients, absence of ammonia and nitrate, etc., the total amount of crop residues would be insufficient for a nitrogen fixation equal to the nitrogen demands of the wheat crop. As mentioned above, we may reckon with a total production of some 130 lb. straw and root residue per bushel of grain; if the stubble were not burned and the residues utilized with an efficiency as high as in Experiment 4 under high moisture, i.e. 3 parts of nitrogen per 1000 parts of residue, the resulting gain would only be about 0·4 lb. nitrogen compared with the consumption of 1·2 lb. nitrogen per bushel of grain. If the stubble were burned (which is the usual practice, and necessary before short fallowing or sowing on stubble-land) there would only be 60–80 lb. residue left to give a fixation of some 0·2–0·25 lb. N compared with a consumption of about 1·5 lb. N per bushel of grain plus burned straw.

About one-third of the nitrogen content of the grain must therefore be considered the highest yield that could be expected from nonsymbiotic nitrogen fixation on the basis of the crop residues, and this only under conditions that are not commonly fulfilled in Australian wheat soils.

There is no reason to think that the residues of roots and stubble left by oats crops for hay or grazing will be qualitatively or quantitatively much different from the residues of wheat.

Generally speaking, it becomes difficult to uphold the belief in the high importance of non-symbiotic nitrogen fixation in arid soils under permanent cereal cultivation, as maintained by several American and Indian investigators (see introduction). To give a complete compensation for the nitrogen consumption represented by the grain alone (1·2 lb. per bushel) the nitrogen-fixing bacteria would have to fix approximately 10 parts of nitrogen per 1000 parts of total residue (130 lb. straw and roots per bushel of grain). This is very close to the yield in vigorous mixed cultures of nitrogen-fixing and cellulose-decomposing bacteria under laboratory conditions, and is difficult to reconcile with the results of the present experiments, which have shown so consistently that cellulosic materials serve far better as food material for nitrogen fixation under high than under low degrees of moisture.

As previously mentioned, there is no real proof that other organisms than *Azotobacter* will fix significant quantities of nitrogen under arid soil conditions. Neither is there any actual foundation for the belief that *Azotobacter* will utilize its energy material far more economically in the soil (and in arid soils especially) than in pure cultures. This appears from those experiments in the present series where nitrogen fixation was most intensive, viz. Experiments 2*a*, 4 and 5 with extra additions of small amounts of glucose or salts of organic acids.

Experiment No.	Gain of Nitrogen, mgm.	
	Per gm. of Substance.	Per gm. of Org. Carbon.
2 *a*, Glucose (av. *a* and *b*)	15·4	38·5
5, Glucose (av. *a* and *b*)	14·1	35·3
5, Ca-lactate (av. *a* and *b*)	5·96	18·6
5, Ca-acetate (av. *a* and *b*)	5·3	17·8
4, Ca-acetate (av. *a* and *b*)	3·8	12·8

The conditions for the activity of *Azotobacter* were here in every respect optimal: moderate moisture content, free access of oxygen, optimal temperature, low concentration of organic nutrients, adequate supply of calcium carbonate, phosphate and molybdenum, absence of nitrate. If the fixation takes place in field soils with the same economy as in these experiments, the annual gain of 20–40 lb. N per acre, so frequently referred to, will require the consumption by *Azotobacter* of at least 550–1100 lb. carbon in compounds equal to glucose in nutritive value, or twice this amount in compounds like lactic or acetic acid. Rather than in cultivated soils from which crops are continually carried away, such supplies of organic nutrients could be expected under prairie conditions (cf. Hall, 1905*a*) or in the leaf beds of forests (Olsen, 1932), where large quantities of plant material of a wide C/N ratio are allowed to decompose *in situ*. And also in these cases a high degree of moisture must be considered essential for an effective utilization of the cellulosic materials for nitrogen fixation, whether by *Azotobacter* or by butyric acid bacilli. Investigations on the numbers of nitrogen-fixing bacteria arising under such conditions would be a matter of great interest.

4. *Soils Exposed to Daylight.*

Now that we have seen that neither the native soil "humus" nor the crop residues provide sufficient energy material for a nitrogen fixation of any significance in comparison with the nitrogen demands of the crops, we are left with the algae as a last possible factor in non-symbiotic nitrogen fixation.

That the growth of algae may result in the addition of considerable quantities of nitrogen to soil or sand media has been shown by several investigators, of whom we need mention only Schloesing and Laurent (1892), Richter (1899), Bouilhac and Giustiniani (1901), and Wilfarth and Wimmer (1907). It has also been shown repeatedly (for references, see Waksman, 1932, and Bristol and Page, 1923) that *Azotobacter* can live in symbiosis with green algae and fix nitrogen at the expense of organic compounds elaborated by these. Some authors (e.g. Koch, cit. after Pfeiffer et al. (1910), Wilsdon and Ali (1922) and Gainey (1930) with special reference to semi-arid cultivated soils) regard this function of the algae as an important link in the nitrogen metabolism of the soil, but these statements are based more on conjecture than on quantitative experimental data. Others, e.g. Pfeiffer et al. (1910), have expressed grave doubts as to whether the, at the best, very scanty algal growth observed on cultivated soils could ever be of any importance in comparison with the luxuriant growth that is produced where even a moderate quantity of nitrogen is fixed.

No investigations have yet been carried out on the occurrence of algae in Australian wheat soils, but theoretically the prospects of a significant gain of nitrogen through the combined action of algae and nitrogen-fixing bacteria do not

seem favourable. The quantity of algal substance in the soil can only be very roughly guessed (Russell, 1937), and since the rates of death and reproduction of algal cells in the soil are unknown, we cannot, even if improved methods of counting gave us a reliable estimate of the quantity of algal matter at a given moment, calculate the amount of substance produced and again transformed in a given interval of time. But if we assume that 50% of the dry matter of the algal substance becomes available to *Azotobacter* and is used for the fixation of 20 mgm. nitrogen per gm. of organic matter, a simple calculation shows that even the moderate fixation of 10 lb. nitrogen per acre per annum will require the production of 1000 lb. dry algal matter per acre annually—a figure equal to the weight of the straw of a 10-bushel wheat crop. Such a production appears unlikely on the wheat soils, where the growth of algae is never conspicuous. To the weight of the actual cell material we should indeed add the amount of organic matter secreted by the cells during growth, but this amounts to, at the most, 30% of the cell material, and may be much less (Roberg, 1930). Our assumption of a fixation of 20 mgm. N per gm. of organic matter implies that it is all utilized by *Azotobacter* working at almost maximal efficiency; this condition can more easily be fulfilled in pure cultures than in the soil, where other organisms will compete with *Azotobacter* for the food material, particularly in the presence of ammonia or nitrate. Finally, not all the soluble compounds derived from the algae may be favourable nutrients for nitrogen fixation by *Azotobacter*; as shown by Aleyev (1934), autolysis products of algae contain certain quantities of amino-nitrogen which might interfere with the nitrogen fixation, either directly or after being broken down to ammonia or nitrate (cf. also De, 1939). Whether other organisms can assimilate nitrogen in association with algae is not known, but it seems unlikely that highly efficient organisms of this kind, if existing, should still have escaped detection. Gains of nitrogen in this way can thus only be expected in soils favourable for *Azotobacter*.

Besides through the association of algae and *Azotobacter*, nitrogen may be fixed directly by certain blue-green algae. Very little is yet known about the distribution and ecology of these organisms. If they are of common occurrence in the wheat soils, their function might possibly be of some importance. Provided the dry cell substance has a nitrogen content of 5% (which is probably rather high), a fixation of 10 lb. nitrogen per acre would involve the production of 200 lb. dry algal matter, which is not in itself an unreasonable figure, and which might not be conspicuous to the naked eye, especially if photosynthesis could take place not only on the surface, but also in the depth of the soil. It has recently been claimed by Fehér and Frank (1936*a*) that blue-green algae are capable of utilizing infra-red radiation penetrating into the deeper layers of the soil, so that photosynthesis may take place well below the soil surface. If this is the case, nitrogen fixation might conceivably also occur. This theory, however, is not supported by the earlier experiments of Schloesing and Laurent (1892) and Wilfarth and Wimmer (1907), where practically all the fixed nitrogen was present in a thin external layer of sand. The observations of Drewes (1928) are even more decisive: cultures of nitrogen-fixing blue-green algae failed to fix nitrogen under a simple cover of black paper, while even cardboard, according to Fehér and Frank (1936*a*), lets through sufficient infra-red radiation for photosynthesis. For the present we can therefore only reckon with nitrogen fixation by algae (directly or in association with bacteria) on the actual soil surface. If such a gain is observed in a laboratory experiment and we wish to express it in

terms of pounds per acre of soil, we must convert it on the basis of area and not of weight of soil.

A number of soils from Table 2 were tested for a possible gain of nitrogen through such processes; 12 wheat soils were used, besides soil No. 8 with 0·2% $CaHPO_4$, which had been found to fix nitrogen on addition of glucose. Portions of 80 to 200 gm. of air-dry soil were moistened to approximately two-thirds of their water-holding capacity and placed for a period of 3–3½ months in an attic window facing the east. No gas was burned and no chemicals used in this room, in order to minimize the danger of absorption of nitrogen compounds from the air. Petri dishes were at first used as containers for the soil, but were later replaced by 300 to 500 c.c. conical suction flasks with the neck closed with a well-fitting rubber stopper and the side tube filled with a loose plug of cotton wool. By this arrangement the atmospheric air had free access to the soil, while the evaporation of moisture, which in sunshine was undesirably strong from the Petri dishes, was reduced to a minimum. After incubation, total nitrogen was determined in the soil, and tests for *Azotobacter* and determinations of ammonia and nitrate were made in some cases. Most experiments were carried out on the same material from which the samples for the nitrogen fixation experiments without organic matter, with glucose and with straw, had been taken. As in the glucose and straw experiments, the average of all the nitrogen determinations on soil without addition of organic matter was taken as the initial content to be compared with the nitrogen content of the sample exposed to daylight. In some cases, however (Nos. 21, 23, 29 and 30), the experiment was carried out with soil that had been washed free from nitrate after incubation for 30 days at 28–30°C. By thus starting with nitrate-free soil it was hoped to enhance the chances of nitrogen fixation. Soil No. 19 was used both untreated and after incubation and washing; in the latter case it was also given an addition of 1·0% $CaCO_3$ and an inoculum of 1·0% of soil No. 8, which by then had been found to fix nitrogen in the daylight. Supplementary experiments were carried out on 3 soils with additions of small quantities of oats-straw, and on almost pure sand inoculated with soil No. 8 after incubation.

The growth of algae during the incubation was in most cases rather scanty, consisting of a few greenish or bluish-green specks of felt-like material, which were often on microscopic examination seen to contain *Oscillatoria*-like organisms; a few moss-protonemas were also sometimes seen. In some cases (Nos. 20 and 24) algal growth was not even visible. The only exceptions from the general rule were soil No. 8 and the sand inoculated therewith. The soil had on its surface quite a heavy brownish to bluish-green algal growth (see fig. 8, Pl. i), in some places forming actual gelatinous drops, and the outer layers of the sand were covered with a similar bluish-green layer. In two soils (No. 6 and No. 19 + $CaCO_3$) a few small plants of *Poa annua* developed during incubation; at the conclusion of the experiment these were separated carefully from the soil and analysed separately for nitrogen.

The results of the nitrogen determinations are shown in Table 25. A significant gain of nitrogen has only taken place in soil No. 8, where it amounts to about 6% of the initial content, and in the sand inoculated herewith. Soil No. 12 shows a definite loss of nitrogen, and the same appears at first glance to be the case in Nos. 6 and 19 + $CaCO_3$, but when allowance was made for the nitrogen found in the grass plants that had developed here, the losses were seen to be non-significant.

Table 26 shows that in No. 8 and the sand, where nitrogen fixation had taken place, all mineral nitrogen had disappeared during the incubation. In all other soils without addition of straw, some mineral nitrogen was present after incubation; in Nos. 29 and 30 there is even a strong nitrification, corresponding to about 4·5–5·5% of the total organic nitrogen. The additional experiments with addition of straw were made in order to prevent the accumulation of nitrate from interfering with the process of fixation; in view of the experiments in the previous section we may safely assume that no nitrogen fixation would take place on the basis of the straw itself. This treatment certainly results in a permanent removal of the nitrate (although not of all the ammonia), but as seen from Table 25 there is no gain of total nitrogen; indeed, No. 10 shows a loss which is probably significant (cf. Table 14).

These experiments suggest very strongly that algae do not function as an important factor in the nitrogen economy of Australian wheat soils, since there was absolutely no indication of any gain of nitrogen in 12 typical wheat soils after incubation for 3 to 4 months under conditions that may safely be assumed to be more favourable for growth of algae than those obtaining in the fields, where the soil surface for long periods is too dry for algal growth. It is important to note that this was also the case with alkaline soils from which the originally present nitrate had been removed (Nos. 23, 29, and 30, the last two of which were of a highly fertile type), and even where nitrogen-fixing blue-green algae were present, as shown below (No. 19 + $CaCO_3$, nitrate-free and inoculated with soil No. 8), or where provision was made for permanent removal of the nitrate, as in the soils with straw. On the other hand, the results with soil No. 8 and the sand medium demonstrate that the experimental technique used here is satisfactory in so far as it does permit a fixation of nitrogen associated with the growth of algae under certain conditions, which apparently are not fulfilled in the wheat soils. It is probably the presence of large quantities of available phosphate that has stimulated the rich algal growth and nitrogen fixation (cf. Wilfarth and Wimmer, 1907).

The plate counts of *Azotobacter* after incubation are shown at the bottom of Table 25. None of the figures are high, and actually the highest number is found in No. 10 + straw, where a significant loss of nitrogen seems to have taken place. In the two cases where nitrogen fixation has taken place, *Azotobacter* are few in numbers (soil No. 8) or seem quite absent (sand). This suggests strongly that blue-green algae were the primary agents of nitrogen fixation.

A crude culture of a blue-green alga (*Anabaena* sp.) was obtained from the sand by inoculating some of the green matter into Allison and Hoover's (1935) mineral solution (K_2HPO_4 0·5 gm.; $MgSO_4$ 0·2 gm.; NaCl 0·2 gm.; $CaSO_4$ 0·1 gm.; $FeCl_3$ 0·005 gm.; H_2O 1000 c.c.) with 0·005% Na_2MoO_4. Growth could be obtained on a corresponding agar medium, but was here always accompanied by bacterial growth, especially a non-spore-forming motile rod resembling *Bact. herbicola*, the elimination of which did not succeed. After three passages in the liquid medium, however, the culture was free from *Azotobacter* and clostridia, as shown by agar and solution tests, and from aerobic heterotrophic nitrogen-fixing organisms in general (Table 4). A quantitative nitrogen fixation experiment was carried out in the following medium: K_2HPO_4 1·0 gm.; $MgSO_4$ 0·5 gm.; NaCl 0·5 gm.; $CaSO_4$ 0·2 gm.; $FeCl_3$ 0·02 gm.; Na_2MoO_4 0·01 gm.; H_2O 1000 c.c. Portions of 50 c.c. in 300 c.c. Erlenmeyer flasks were sterilized and inoculated each with one loopful of the algal pellicle from a 26-days-old solution culture tested for absence of nitrogen-fixing bacteria. Two flasks were analysed for nitrogen immediately after inocula-

TABLE 25.

Changes in Nitrogen Content of Soils Exposed to Daylight.

Soil No.*	Total N, p.p.m. Mean ($\bar{x}$).	n+1.	$S(x-\bar{x})^2$.	Gain (+) or Loss (−) of N, p.p.m. ($\bar{x}_2-\bar{x}_1$).	t.	P.	Incubation Time.
24. Initial ..	392·7	10	310·1				21/7–7/11,
Inc. 109 d.	393·3	4	242·8	+0·6	0·149	0·9–0·8	1936
8. Initial ..	446·8	11	804·0				14/5–3/9,
Inc. 114 d.	474·0	5	484·0	*+27·2*	5·257	*<0·01*	1936
19. Initial ..	455·2	9	316·2				17/6–28/9,
Inc. 103 d.	459·5	4	137·0	+4·3	1·141	0·3–0·2	1936
12. Initial ..	532·2	10	459·6				8/4–27/7,
Inc. 110 d.	507·3	3	10·7	*−24·9*	5·785	*<0·01*	1936
25. Initial ..	535·7	11	980·6				10/8–23/11,
Inc. 105 d.	527·3	3	74·7	*−8·4*	1·092	0·3–0·2	1936
21. Washed :							
Initial ..	563·3	3	50·7				16/9–21/12,
Inc. 96 d.	558·8	5	162·8	*−4·5*	1·017	0·4–0·3	1936
10. Initial ..	631·9	10	1038·1				21/3–4/7,
Inc. 105 d.	627·0	3	8·0	−4·9	0·763	0·5–0·4	1936
14. Initial ..	649·3	12	2361·5				21/4–11/8,
Inc. 112 d.	653·7	3	20·7	+4·4	0·541	0·6–0·5	1936
20. Initial ..	653·1	11	1394·4				17/7–29/10,
Inc. 104 d.	650·0	5	724·0	−3·1	0·462	0·7–0·6	1936
23. Washed :							
Initial ..	694·6	5	444·8				3/9–11/12,
Inc. 99 d.	689·0	6	716·0	−5·6	0·814	0·5–0·4	1936
6. Initial ..	827·1	11	2528·8				6/2–6/5,
Inc. 90 d.	798·3	3	152·7	*−28·8*†	2·958	*0·02–0·01*	1936
29. Washed :							
Initial ..	948·2	5	544·8				13/10/36–
Inc. 98 d.	953·3	3	20·7	+5·1	0·719	0·5–0·4	19/1/37
30. Washed :							
Initial ..	1050·3	3	2·7				12/10/36
Inc. 95 d.	1044·3	3	60·7	−6·0	1·846	0·2–0·1	15/1/37
19. Washed +$CaCO_3$:							
Initial ..	454·8	4	120·8				26/9–23/12,
Inc. 88 d.	437·3	4	202·8	*−17·5*†	3·370	*0·02–0·01*	1936
Sand :							
Initial ..	36·3	2	3·7				7/12/36–
Inc. 124 d.	55·3	4	25·2	*+19·0*	8·162	*<0·01*	1/4/37
25. +0·6% straw :							
Initial ..	544·5	11	980·6				16/12/36–
Inc. 96 d.	550·3	4	212·8	+5·8	1·037	0·4–0·3	22/3/37

TABLE 25.—*Continued.*

Changes in Nitrogen Content of Soils Exposed to Daylight.—Continued.

10. +0·75% straw:							
Initial ..	642·1	10	1038·1				11/12/36–
Inc. 94 d.	624·7	3	9·7	*−17·4*	2·708	*0·05−0·02*	15/3/37
11. +1·5% straw‡:							
Initial ..	655·0	5	826·0				12/3–30/6
Inc. 110 d.	645·7	4	416·8	−9·3	1·030	0·4−0·3	1937

Azotobacter in soil after incubation: (S+ or S− : presence or absence of *Az.* in mannite sol.).

Soil No.	*Azotobacter* per gram.	Soil No.	*Azotobacter* per gram.
8	55 (S+)	19+$CaCO_3$	0 (S+)
21	0	Sand	0 (S−)
23	380 (S+)	10+straw	1160 (S+)
25	0	11+straw	370 (S+)
29	270 (S+)	25+straw	0

* Soils No. 24, 8, 19, 12, 25, 10, 14 and 6 were incubated in Petri dishes, the rest in flasks.

† Small plants of *Poa annua* developed during incubation.

‡ After incubation aerobically for 120 days; no nitrate or ammonia present.

TABLE 26.

Mineral Nitrogen in Soils Exposed to Daylight (p.p.m.).

Soil No.	NO_3–N.	NH_4–N.	Soil No.	NO_3–N.	NH_4–N.
24. Initial..	1·1	0	10. Initial..	4·0	4·9
Incubated	16·4	(+)*	Incubated	12·7	0
8. Initial..	3·7	6·8	14. Initial..	26·1	0
Incubated	0	0	Incubated	15·9	(+)
19. Initial..	6·0	4·0	20. Initial..	2·2	0
Incubated	4·5	0	Incubated	15·1	0
19. Washed:			23 Washed:		
Initial..	0	0	Initial..	0	(+)
Incubated	12·4	0	Incubated	(+)	(+)
12. Initial..	4·0	4·0	6. Initial..	11·5	0
Incubated	2·0	0	Incubated	7·7	0
			29. Washed:		
25. Initial..	1·8	4·4	Initial..	0	0
Incubated	24·1	(+)	Incubated	46·3	0
25. +Straw:			30. Washed:		
Initial..	1·8	4·4	Initial..	0	(+)
Incubated	0	6·7	Incubated	56·5	0
21. Washed:			Sand:		
Initial..	0	0	Initial..	0	(+)
Incubated	10·6	0	Incubated	0	0
			10. +Straw:		
			Initial..	4·0	4·9
			Incubated	0	6·2

* (+)=Qualitative test positive.

tion, four were incubated as cultures, and four as controls; two of these were given an addition of 5 c.c. conc. H_2SO_4, and two of 5 c.c. 20% NaOH, in order to serve as controls upon the quantities of ammonia and nitrous oxides, respectively, that might be absorbed from the atmosphere. The cultures were started on 8th April, 1937, and were placed in the same window where the flasks with soil had previously been kept. After 3 to 4 weeks a coherent, slimy, bluish-green pellicle developed; after 6 weeks it did not grow further, and after 8 weeks it had become brownish and rather unhealthy-looking (cf. Winter, 1935). The experiment was therefore discontinued, all cultures tested with a negative result for *Azotobacter* on dextrine agar, and total nitrogen was determined. The results are found in Table 27, first section. The gain of nitrogen in the cultures is quite striking, but neither the acid nor the alkaline control solutions have absorbed the slightest trace of combined nitrogen from the atmosphere. These facts in connection with the absence of nitrogen-fixing bacteria justify the conclusion that the alga itself is capable of nitrogen fixation.

Another crude culture of an alga of similar appearance was later obtained from soil No. 19 + $CaCO_3$. This strain was also found free from *Azotobacter* and clostridia, and was tested for nitrogen fixation in the same way, but the medium was made up with tap water, which had been observed to give a more healthy-looking growth than the distilled water. The results are given in the second section of Table 27. Practically no growth took place during the first 6 weeks of incubation (July-August) when a low temperature prevailed; the rise in temperature in September was accompanied by development of dense bluish-green pellicles in all culture flasks. The control flasks, one of which had been boiled for a few seconds after inoculation instead of receiving an addition of sulphuric acid, give some indication of a slight, perhaps not significant, absorption of nitrogen from the atmosphere. The cultures in this series were also free from *Azotobacter* at the end of the experiment; they all show gains of nitrogen, which upon the whole are higher but less consistent than in the first series. Culture *a*, which had fixed least nitrogen, also showed the smallest amount of growth. (Fig. 9, Pl. i, shows the appearance of the organism).

There is thus no doubt that organisms of this group under certain circumstances can assimilate elementary nitrogen in such quantities as to give a measurable increase in the nitrogen content of the soil, such as in soil No. 8 and the sand culture in Table 24. But it is not permissible to apply the results obtained from these media of alkaline reaction,[34] plentiful supply of available phosphate, and a surface which has artificially been kept moist and thus favourable for algal growth for 3–4 months, to the wheat soils which even under similar external conditions do not produce such a conspicuous growth of algae, which are very often of an acid reaction, in which the soil surface under field conditions is moist for short periods only (except perhaps in winter time when the temperature conditions are unfavourable; according to Allison and Hoover (1935), the optimal temperature for nitrogen-fixing blue-green algae is 28–30°C.), and where the almost constant presence of smaller or larger quantities of nitrate (Prescott, 1934; Penman and Rountree, 1932), could hardly fail to interfere with the process of fixation (De, 1939). But even if there were wheat soils where reaction and supply of mineral nutrients were adequate, and where once every year the concentration of assimilable mineral nitrogen (ammonia and nitrate) were sufficiently low and

[34] The algae studied by Allison and Hoover (1935) and Winter (1935) had optimum at pH 7-8; little or no N was fixed at pH 6·0-6·5.

TABLE 27.

Nitrogen Fixation in Cultures of a Blue-green Alga.

Ser. I. Incubated 8 weeks (4/5/37–19/6/37) in daylight.

Control Solutions.	Total N. Mgm. per Flask.	Cultures.	Total N. Mgm. per Flask.
Initial (*a*)	0·06	(*a*)	1·22
(*b*)	0·11		
Inc.+H_2SO_4 (*a*)	0·02	(*b*)	1·33
(*b*)	0·03		
Inc.+NaOH (*a*)	0·04	(*c*)	1·34
(*b*)	0·03	(*d*)	1·42
Average of controls	0·05	Average of cultures	1·33

Average gain of N per culture: 1·28 mgm.

Ser. II. Incubated 10 weeks (18/7/38–26/9/38).

Control Solutions.	Total N. Mgm. per Flask.	Cultures.	Total N. Mgm. per Flask.	Gain of N. Mgm. per Flask.
Initial (*a*)	0·05	(*a*)	1·25	1·15
(*b*) ..	0·05			
Incubated, boiled ..	0·06	(*b*)	1·92	1·82
Incubated+H_2SO_4	0·11	(*c*)	2·14	2·04
Incubated+NaOH (*a*)	0·19	(*d*)	1·94	1·84
(*b*)	0·12			
Average of controls..	0·10	Average gain		1·71

the moisture conditions sufficiently favourable to allow the formation over the whole field of a continuous sheet of nitrogen-fixing blue-green algae as heavy as that observed in the second experiment in Table 27, the resulting gain of nitrogen would be inconsiderable. The fixation amounted in this experiment to 1·15 to 2·04 mgm. per flask, in which the surface area of solution was approximately 50 cm^2. Computed on the basis of an acre of soil, this would correspond to 2·1 to 3·6 lb. per acre, which is only equivalent to the nitrogen content of two to three bushels of wheat grain.[35]

While we may thus agree with Pfeiffer et al. (1910) in concluding that the activity of algae cannot be credited with any importance in the wheat soils, it cannot be denied that these organisms may play a significant role under different conditions, for instance in freshwater lakes or in rice fields where a rich growth of algae is frequently produced during the period of water-logging (references by Russell, 1937). De (1936) observed a rich development of algae and vigorous fixation of nitrogen in suspensions of rice soils exposed to daylight, and later (1939) he isolated N-fixing blue-green algae therefrom. The classical example of growth of blue-green algae on bare volcanic soils, where a nitrogen reserve may slowly be built up through the activity of these organisms, is too well known to

[35] A similar calculation might be applied to soil No. 8 in Table 25. The actual gain of nitrogen was 2·0 ± 0·403 mgm. per 74·5 gm. dry soil in a Petri dish with internal diameter 9 cm., surface area of the soil consequently 64 $cm.^2$ On the basis of an acre of soil the gain would only correspond to 2·8 ± 0·56 lb. of nitrogen.

need comment; it is highly probable that primary nitrogen fixation by blue-green algae is responsible for much of this gain.

It may finally be mentioned that the results found with soils + straw exposed to daylight in Table 25 give no indication that the straw is utilized for photochemical nitrogen fixation, as suggested by Dhar (1937) on the basis of experiments with soil + cellulosic materials exposed to sunlight. It is true that ultraviolet radiation was almost completely excluded, but this seems also to have been the case in Dhar's experiments, and in any case this kind of radiation could not act upon organic matter incorporated in the soil, since it does not penetrate below the actual soil surface (Fehér and Frank, 1936*a*). Moreover, the possible effect of algae does not seem excluded in Dhar's experiments.

(e) Mineralization of Humus Nitrogen in Australian Soils.

The nitrate content of Australian wheat soils has been studied much more exhaustively than the question of the total nitrogen balance (for references, see Prescott, 1934). As mentioned in the introduction, fallowed land is regularly found richer in nitrate than corresponding cropped or stubble land, although these also usually contain certain small amounts of nitrate. Some experiments on the influence of moisture and temperature and the production of nitrate in a wheat soil under laboratory conditions have been carried out in South Australia by Prescott and Piper (1930), but comparative investigations on the ability of a larger number of typical wheat soils to produce nitrate and ammonia from their store of organic nitrogen are yet lacking. A series of experiments was carried out in order to supply this need.

These experiments were carried out in precisely the same way as the nitrogen fixation experiments without addition of organic matter reported in Table 7 (actually these data are nothing but the nitrogen balance in some of the experiments to be described here): duplicate portions (except for a few cases where only small quantities of soil were available) of 150 to 200 gm. of air-dry soil were moistened to about 60% of their water-holding capacity and incubated in large Petri dishes for 30 days at 28–30°C., with determination of nitrate and ammonia before and after incubation. Altogether 55 soils from Table 2 were tested.[30] In some cases the soil that had been extracted with water for nitrate determination was air-dried, re-moistened, and incubated for another period of 30 days. Soil No. 22 was received in a completely water-saturated condition, and was excessively rich in nitrate (54 p.p.m. NO_3–N, or more than 6% of its total nitrogen content); it was therefore air-dried and washed free from nitrate before starting the experiment. The results of this series of experiments are given in Table 28, where the soils are arranged in order of increasing nitrogen content.

All soils, with one exception, produced nitrate during incubation, and the initial content of ammonia, with very few exceptions, decreased or disappeared completely. The exception is represented by the strongly acid, uncultivated soil No. 3 in which, however, a considerable quantity of ammonia accumulated. The increases in mineral nitrogen (nitrate plus ammonia), or "metabolizable nitrogen" to use the term of Richardson (1938), show a very clear correlation with the contents of total nitrogen. A calculation of the correlation coefficient between these two factors shows for all the 106 observations on 55 soils the highly

[30] Some of these represent mixtures of equal parts of two samples from the same field (Nos. 51 + 52, 53 + 54, 55 + 56).

significant value of + 0·818.[37] If we omit the 7 soils outside the wheat belt (Nos. 3, 4, 5, 8, 9, 44, and 55 + 56) as well as the wheat soils Nos. 1 and 2, which had been air-dried for a long period prior to examination and may therefore have given abnormally high results (Waksman, 1932), we have left 90 observations on 46 samples representing normal soils of the wheat district. The correlation coefficient between total nitrogen content and production of $(NO_3 + NH_4)N$ is now reduced to + 0·625, which is still a highly significant value. The last column of the table shows the production of $(NO_3 + NH_4)N$ as percentage of initial *organic* nitrogen (Total N − $(NO_3 + NH_4)N$). This figure, which we might call the "coefficient of mineralization", is seen to vary from 0·14 to 5·18%, the maximum value being found in the long air-dried soil No. 1. In the large majority of the cases (64 out of 106) it lies between 1·5 and 3·0%, generally with good agreement between the two parallel dishes by which most soils are represented. If we take the average of the parallels, we find the following:

Range of Coefficient of Mineralization. Per cent.	Frequency.	
	All 55 Soils.	46 Fresh Wheat Soils.
0–0·5	2	2
0·51–1·0	3	3
1·01–1·5	8	7
1·51–2·0	14	12
2·01–2·5	10	9
2·51–3·0	5	4
3·01–3·5	5	5
3·51–4·0	2	0
4·01–4·5	5	4
4·51–5·0	0	0
5·01–5·5	1	0
Total	55	46

There is here no indication of any excessively rapid nitrification of the humus nitrogen, such as has occasionally been stated to take place in arid and semi-arid soils from North America. For instance, Lipman et al. (1916), in California, found in certain cases up to 50% of the total nitrogen in soil of low humus-content nitrified after 1 month, and Gainey (1936), in Kansas, found that "normal" soils (apart from "fertility spots" with excessive nitrification) could nitrify up to 20% of their nitrogen in 4–6 weeks. There is a much better agreement between the present results and those of similar nitrification experiments by Hall (1922) in South Africa: 54 soils of different character were incubated for 30 days at 28°C., or for 6 weeks at room temperature. Four soils were found incapable of producing nitrate, while in the remaining 50 soils from 0·1 to 5·1% of the total nitrogen was transformed into nitrate, with a clear correlation between the nitrogen content of the soil and the amount of nitrate produced.

There is no obvious correlation between the coefficient of mineralization and general soil type, content of total nitrogen (as also in Hall's experiments), or even hydrogen-ion concentration; indeed, such strongly acid soils as Nos. 4, 5, 7,

[37] Soil No. 35 with extra addition of calcium carbonate has not been included in this calculation.

TABLE 28.

Production of Mineral Nitrogen in Soils Incubated 30 Days at 28–30° C.

Soil No.	Total N. p.p.m.	Before Incubation.			After Incubation.			$(NO_3+NH_4)N$ produced.	
		NO_3–N p.p.m.	NH_4–N p.p.m.	NO_3–+ NH_4–N p.p.m.	NO_3–N p.p.m.	NH_4–N p.p.m.	NO_3–+ NH_4–N p.p.m.	p.p.m.	% of Initial Organic N.
31	183	1·8	3·5	5·3	11·6 11·5	2·0 1·5	13·6 13·0	8·3 7·7	4·66 4·33 } 4·5
33	228	3·8	3·4	7·2	9·0 7·5	0 0	9·0 7·5	1·8 0·3	0·81 0·14 } 0·5
32	243	1·8	5·8	7·6	9·3 7·6	3·9 2·8	13·2 10·4	5·6 2·8	2·37 1·19 } 1·8
24	393	1·1	0	1·1	8·5 8·4	0 0	8·5 8·4	7·4 7·3	1·89 1·86 } 1·9
72	428	0·5	8·4	8·9	27·1 22·0	1·8 2·1	28·9 24·1	20·0 15·2	4·76 3·62 } 4·2
*8	447	3·7	6·8	10·5	19·5 17·7	0 0	19·5 17·7	9·0 7·2	2·07 1·63 } 1·8
19	455	6·0	4·0	10·0	22·0 22·4	0 0	22·0 22·4	12·0 12·4	2·70 2·78 } 2·7
12	533	4·0	4·0	8·0	16·6 16·4	0 0	16·6 16·4	8·6 8·4	1·64 1·60 } 1·6
25	536	1·8	4·4	6·2	22·4 22·0	1·4 3·1	23·8 25·1	17·6 18·9	3·32 3·56 } 3·4
58	556	0	0	0	18·1 17·2	0 0	18·1 17·2	18·1 17·2	3·25 3·09 } 3·2
57	563	0	2·6	2·6	15·6 14·9	0 0	15·6 14·9	13·0 12·3	2·32 2·19 } 2·3
21	583	5·3	2·7	8·0	11·9 12·6	0 0	11·9 12·6	3·9 4·6	0·68 0·83 } 0·8
37	621	5·4	2·8	8·2	32·9 31·4	1·8 2·1	34·7 33·5	26·5 25·3	4·32 4·13 } 4·2
10	632	4·0	4·9	8·9	19·9 19·2	0 0	19·9 19·2	11·0 10·3	1·76 1·65 } 1·7
11	634	6·4	5·4	11·8	24·6 22·3	3·4 0	28·0 22·3	16·2 10·5	2·60 1·69 } 2·1
13	643	4·3	5·3	9·6	30·0 32·3	0 0	30·0 32·3	20·4 22·7	3·22 3·58 } 3·4
14	649	26·1	0	26·1	31·1 32·4	0 0	31·1 32·4	5·0 6·3	0·80 1·01 } 0·9

TABLE 28.—*Continued.*

Production of Mineral Nitrogen in Soils Incubated 30 Days at 23–30° C.—Continued.

53+54	650	0·7	0	0·7	13·6 13·7	0 0	13·6 13·7	12·9 13·0	1·98 2·00 } 2·0
20	653	2·2	0	2·2	13·7 14·5	0 0	13·7 14·5	11·5 12·3	1·77 1·89 } 1·8
38	664	6·5	4·2	10·7	24·5 24·8	2·2 1·6	26·7 26·4	16·0 15·7	2·45 2·40 } 2·4
*3	688	0	5·9	5·9	0 0	22·9 21·4	22·9 21·4	17·0 15·5	2·49 2·27 } 2·4
35	715	5·1	8·1	13·2	16·9 15·7	0 0	16·9 15·7	3·7 2·5	0·53 0·36 } 0·4
35+1% $CaCO_3$	711	5·1	8·1	13·2	36·0 37·5	2·0 0	38·0 37·5	24·8 24·3	3·55 3·48 } 3·5
49	721	3·5	4·0	7·5	17·6 15·9	2·0 0·9	19·6 16·8	12·1 9·3	1·70 1·30 } 1·5
7	732	23·0	0	23·0	45·9 51·6	10·8 0	56·7 51·6	33·7 28·6	4·75 4·03 } 4·4
34	745	9·4	10·9	20·3	34·9 31·7	0 0	34·9 31·7	14·6 11·4	2·01 1·57 } 1·8
23	749	4·3	0	4·3	26·8 25·4	0·7 1·1	27·5 26·5	23·2 22·2	3·11 2·97 } 3·04
15	755	25·4	5·7	31·1	39·5 36·8	0 3·0	39·5 39·8	8·4 8·7	1·16 1·20 } 1·2
27	790	3·2	3·7	6·9	21·7 20·5	0 0	21·7 20·5	14·8 13·6	1·89 1·74 } 1·8
50	795	1·7	0	1·7	16·9 17·2	0 0	16·9 17·2	15·2 15·5	1·92 1·96 } 1·9
1	798	9·6	0	9·6	50·4	0	50·4	40·8	5·18 5·2
39	798	9·2	3·8	13·0	32·0 32·0	1·7 0	33·7 32·0	20·7 19·0	2·64 1·74 } 2·2
22	813	0	13·0	13·0	28·8 34·2	3·0 2·1	31·8 36·3	18·8 23·3	2·35 2·91 } 2·6
51+52	816	1·1	0	1·1	20·0 16·2	0 0	20·0 16·2	18·9 15·1	2·32 1·85 } 2·09
6	827	11·5	0	11·5	25·9 27·5	0 4·6	25·9 32·1	14·4 20·6	1·77 2·53 } 2·2
73	836	0·4	6·5	6·9	23·2 23·5	1·2 0	24·4 23·5	17·5 16·6	2·11 2·00 } 2·1
71	933	18·4	2·9	21·3	26·6	0	26·6	5·3	0·58 0·6
70	961	2·8	2·6	5·4	18·7	0	18·7	13·3	1·39 1·4

TABLE 28.—*Continued.*

Production of Mineral Nitrogen in Soils Incubated 30 Days at 28–30° C.—Continued.

Soil No.	Total N, p.p.m.	Before Incubation.			After Incubation.			$(NO_3+NH_4)N$ produced.	
		NO_3–N p.p.m.	NH_4–N p.p.m.	NO_3–+ NH_4–N p.p.m.	NO_3–N p.p.m.	NH_4–N p.p.m.	NO_3–+ NH_4–N p.p.m.	p.p.m.	% of Initial Organic N.
29	964	9·3	1·7	11·0	26·2 26·0	0 0	26·2 26·0	15·2 15·0	1·59 1·57 } 1·6
36	985	26·2	11·9	38·1	72·3 68·0	1·8 0	74·1 68·0	36·0 29·9	3·80 3·16 } 3·5
69	986	0	6·7	6·7	24·8 22·6	0 0	24·8 22·6	18·1 15·9	1·85 1·61 } 1·7
68	1053	0	0	0	22·3 22·8	0 0	22·3 22·8	22·3 22·8	2·12 2·14 } 2·13
30	1072	8·4	1·5	9·9	27·2 25·7	1·5 1·9	28·7 27·6	18·8 17·7	1·77 1·74 } 1·8
*55+ 56	1152	7·6	5·8	13·4	33·6 33·2	0 0	33·6 33·2	20·2 19·8	1·77 1·74 } 1·8
2	1257	10·9	0	10·9	61·0	0	61·0	50·1	4·01 4·0
26	1265	6·5	0	6·5	32·9 32·9	1·5 2·7	34·4 35·6	27·9 29·1	2·24 2·30 } 2·3
66	1508	5·2	7·0	12·2	43·2 40·5	2·4 0	45·6 40·5	33·4 28·3	2·23 1·89 } 2·1
28	1573	3·9	4·4	8·3	26·8 26·3	1·7 1·5	28·5 27·8	20·2 19·5	1·29 1·24 } 1·3
16	1604	11·3	4·0	15·3	33·3 29·7	0 0	33·3 29·7	18·0 14·4	1·13 0·96 } 1·0
*44	1728	0	3·6	3·6	27·9 28·8	0 0	27·9 28·8	24·3 25·2	1·41 1·46 } 1·4
64	1822	2·5	0	2·5	23·5 20·0	0 0	23·5 20·0	21·0 17·5	1·15 0·96 } 1·1
17	1900	12·1	5·9	18·0	34·7 45·2	5·9 5·9	40·6 51·1	22·6 33·1	1·20 1·76 } 1·5
18	2060	46·1	6·7	52·8	94·4 89·9	2·7 3·0	97·1 92·9	44·3 40·1	2·21 2·00 } 2·11
*5	2710	24·4	14·7	39·1	135·4 125·3	8·4 6·7	143·8 132·0	104·7 92·9	3·92 3·48 } 3·7
*4	3280	45·9	30·8	76·7	207·3 166·6	7·6 7·7	214·9 174·3	138·2 97·6	4·32 3·05 } 3·7
*9	5860	20·7	10·2	30·9	189·2 194·0	5·8 6·0	195·0 200·0	164·1 169·1	2·82 2·91 } 2·9

TABLE 28.—*Continued.*

Production of Mineral Nitrogen in Soils Incubated 30 Days at 28–30° C.—Continued.

Second period of 30 days.

58	556	0	0	0	13·0	0	13·0	13·0	2·34
14	649	0	0	0	9·7	0	9·7	9·7	1·56
15	755	0	0	0	12·2	0	12·2	12·2	1·68
71	933	0	0	0	5·1	0	5·1	5·1	0·56
70	961	0	0	0	9·3	1·9	11·2	11·2	1·17
64	1822	0	0	0	16·9	0	16·9	16·9	0·93

Soils marked * are outside the wheat belt.

36 and 58, of pH 4·7 to 5·5, have shown a remarkably vigorous production of mineral nitrogen practically all consisting of nitrate. It is interesting to note that the three soils from Rutherglen Exp. Farm, Vic., viz., Nos. 25, 36, and 37, are among the most vigorously nitrifying soils and transform 3·2–4·3% of their organic nitrogen into nitrate in 30 days; the particular aptitude for nitrification which these soils seem to possess may be the explanation why nitrogenous fertilizers have always at this locality failed to increase the yield of wheat even when sown on stubble land. This circumstance deserves attention in future field trials with nitrogenous fertilizers.

The appendix to Table 28 shows that the nitrate produced during the first 30 days of incubation does not represent a separate, especially rapidly decomposible proportion of the humus nitrogen; when it is removed by washing and the soil is re-incubated, the mineralization goes on at about the same rate as in the first period. By prolonged incubation it is probable that the process would slow down (cf. Russell and Richards, 1920; Fraps, 1920).

The conditions under which the soil samples were kept in these experiments are comparable with those obtaining in fields under bare fallow in summer time with good conservation of the moisture. The experimental results justify the conclusion that under this treatment all normal Australian wheat soils may be expected to convert a smaller or larger percentage of their humus nitrogen into nitrate ready to be taken up by the subsequent wheat crop. The very significant correlation between production of mineral nitrogen and content of total nitrogen in the nitrification experiments suggests that in soils under equal treatment we may expect the quantity of nitrogen produced during fallow to be roughly proportional to the humus content of the soil. Richardson and Gurney's (1934) explanation for the ineffectiveness of nitrogenous fertilizers on fallowed land, viz., the higher nitrate content in this than in stubble land, would thus seem to have a general application. But higher crop yields after fallowing do not necessarily mean that any actual "recuperation" of the fertility of the soil has taken place—they may equally well indicate stronger exploitation of the nitrogen resources of the soil (cf. Pfeiffer, 1904).

PRECIPITATION AND LEGUMINOUS PLANTS AS SOURCES OF NITROGEN.

We have now seen that non-symbiotic nitrogen fixation can never be expected to cover the whole nitrogen consumption of the wheat crops, and only under exceptional circumstances a small fraction of it, while in the average wheat soils under the system of cultivation usually adopted in New South Wales it cannot even be assumed that any nitrogen will be gained through this process at all.

But before we try to form a general picture of the nitrogen economy of the wheat soils, we must consider the existence of certain other factors counterbalancing the loss of nitrogen due to cultivation.

The first of these is the rain water, which is known to contain certain amounts of combined nitrogen, chiefly as nitrous oxides produced by electric discharges in the atmosphere. Data on amounts of nitrogen annually added to the soil by rain in various parts of the world have been collected by Miller (1913), but the question has not yet been studied systematically in Australia. The only observations here are due to Brünnich (cit. after Miller, 1913), who carried out analyses of the rain water in three localities in Queensland; one of these—Roma—was within the wheat district. The amount of nitrogen annually brought down by the rain was estimated at 3·1 to 4·1 lb. per acre, a figure very similar to that which has been found in other parts of the world away from industrial centres. It may therefore be a conservative estimate to reckon with an annual gain of 3 lb. nitrogen per acre from the rain. While this is insignificant in districts where the average yield of wheat is as high as 15–20 bus., it may be relatively important where the average yield is as low as 8–10 bus., particularly if a crop is taken only every second year (an 8 bus. crop would consume little more than 12 lb. N per acre, half of which might thus be compensated by two years' rainfall). In no case, however, can we consider this more than a contributing factor in maintaining the nitrogen supply of the soil.

A far more important factor is the symbiotic nitrogen fixation by leguminous plants. On the areas devoted to wheat cultivation in New South Wales legumes are rarely sown, except where the land is laid down to pasture, usually with mixtures of grasses and clovers, or with lucerne. On the other hand, growth of self-sown clovers and trefoils is common on land under the usual wheat-fallow or wheat-oats-fallow rotation, and especially on land left as pasture after cereal crops. Howell (1911) called attention to this phenomenon in Victorian wheat soils, and mentioned the possibility that it might suffice to cover the losses of nitrogen caused by wheat cultivation. According to Breakwell (1923), the most common wild legumes in the wheat district of New South Wales are Burr trefoil (*Medicago denticulata*) and Ball clover (*Trifolium glomeratum*), which may occur both in the wheat crops and among the herbage on the stubble fields, but reach their richest development on land left undisturbed for a couple of years after fallowing for two or three wheat crops. Breakwell mentions this practice as most successful in improving the yields of wheat in the south-western and Riverina districts of New South Wales, and suggests the sowing of these species on such laid-out land. No systematic work on the distribution and density of these plants on the wheat lands of New South Wales has yet been undertaken. An inquiry among various field officers of the Field Branch of the Department of Agriculture of N.S.W. in March–April 1938[38] elicited the general answer that where moisture conditions had permitted a growth of clovers and trefoils, it was mainly confined to the better types of soil and appeared generally to be stimulated by the use of phosphatic fertilizers, but did mostly not become heavy except on undisturbed land (Riverina, cf. Breakwell, 1923), since the winter-ploughing of the fallows tends to check the growth. In the northern districts, where short fallowing is the general rule, fair growth was sometimes reported in the wheat crops, especially on black soils in the New England–Inverell districts; an abundance of *Medicago denticulata*, *lupulina* and *maculata* were said to occur among the crops here.

[38] Through the courtesy of Mr. H. C. Stening, Chief Agricultural Instructor, Department of Agriculture, N.S.W.

The importance of these plants in adding nitrogen to the soil is difficult to estimate.[39] We have no knowledge of the actual proportion of the wheat area where wild legumes occur at all, nor any quantitative data on the mass of growth that they produce where they occur. Further, their development varies greatly with the season, and fields where they grow well are usually grazed by sheep, so that not all the legume-nitrogen is returned to the soil; other complicating factors are the proportion of nitrogen taken from soil and from the atmosphere (which may vary greatly with the conditions of growth, and especially the nitrate content of the soil), as well as the problem whether the legumes may actually by root-secretion supply nitrogen directly to the wheat plants when occurring in the wheat crops.

All these problems (distribution and numerical representation of wild legumes, production of organic matter, capacity for nitrogen fixation, possible nitrogenous root secretion, etc.) need systematic and intensive study.

Until this has been undertaken, we can only say in a general way, that where spontaneous growth of legumes takes place, it is likely to add some nitrogen to the soils, but this remains an unknown quantity, and in ordinary cropping systems with alternating cereals and fallow it is more than unlikely that it would suffice to cover the nitrogen demands of the crops, especially since the legumes mainly appear on the better type of lands, where a comparatively high yield of wheat may be expected. If the land is worked on a short-fallow system, the growth of the legumes is almost confined to the crops, where their nitrogen fixation may be considerable, but of the quantitative aspect of this we can form no opinion. Under long-fallowing, which is common in the southern parts of New South Wales with summer minimum of rainfall, germination of the legumes on the stubble fields usually starts with the autumn rains, and the plants do not reach any considerable size before they are ploughed under in the early winter and thus do not reach the period where most nitrogen is fixed; in this case also grazing is common. Here, as before, it holds that we can form no idea *a priori* of the possible importance of legumes growing in the wheat crops.

Where legumes are sown or where wild legumes produce a good growth on land allowed to revert to pasture for a year or two, things may of course be entirely different; but it must be left to future investigations (which are urgently needed) to inform us on the gains of nitrogen that may be expected here.

General Conclusions.

Where soil erosion has not set in, the nitrogen consumption of the cereal crops must be regarded as the chief source of loss of nitrogen. Actual leaching of nitrate by rains appears unlikely, although the nitrate may be washed into the subsoil (whether the disappearance of nitrate, which according to Penman and Rountree (1932) may take place here, represents an actual loss or merely a transformation of nitrate, remains to be decided). On this assumption we may try to estimate the loss of soil nitrogen under wheat cultivation.

i. If wheat, or wheat followed by oats, is grown alternately with bare fallow, long or short, the resulting loss of nitrogen to the soil may, apart from exceptional cases where it is to a small extent offset by non-symbiotic fixation, be expressed simply as (N in grain + N in straw burned or harvested as hay) — (N in seed + N in rain falling during the rotation).

[39] Few attempts have been made to estimate the gains of nitrogen by wild legumes. Alway and Pinckney (1909) suggested (very approximately) an annual gain of 8 lb. N per acre under prairie conditions.

This difference, which in normally yielding fields will always be positive,[40] must be covered by nitrogen taken from the soil's store of humus. In the simplest case—wheat alternating with long fallow—an average wheat crop of 12 bus. per acre every second year might thus be estimated to cost the soil:

N in grain	14 lb.
N in burned straw	4 lb.
Sum	18 lb. N per acre

offset by:

N in seed	1 lb.
N in two seasons' rain 2 × 3 = ...	6 lb.
Sum	7 lb.

i.e. a net loss of 11 lb. N per crop, or 5·5 lb. per acre per annum. This is equivalent to the nitrogen content of 96 lb. soil organic matter, if we reckon with a C/N ratio of 10:1 and a factor of 1·75 for conversion of carbon into total organic matter (Waksman, 1932). With increasing yield the balance becomes increasingly unfavourable, owing to the proportionately smaller offset by nitrogen in seed and rain.

ii. Under a similar system of cultivation, but where legumes appear on fallowed land or among crops, the loss of nitrogen may be somewhat further compensated by the legumes, largely dependent on the growth that these plants make, but it is impossible to tell beforehand whether this compensation will be complete.

iii. If the cereal crops alternate with pastures where leguminous plants succeed well, it is possible, but by no means certain, that the nitrogen fixation by the legumes will be sufficient to cover the nitrogen consumption by the cereal crops (among other things, this will depend on the ratios between the quantities of organic matter produced by cereals, grasses, and legumes, as well as upon the extent to which the pastures are grazed).

Examples i and ii represent the most common state of affairs in New South Wales. We may conclude that the former case certainly, and the latter probably, represents a gradual spending of the nitrogen reserves of the soil or a "depletive cultivation",[41] which is rendered possible through the decomposition of a part of the humus resulting in nitrate production, especially under fallow, but which will ultimately lead to impoverishment of the soil through simple lack of nitrogen, if it has not before then resulted in erosion. It is a common experience that the tendency to this phenomenon arises after a shorter or longer period of cultivation and consequent alteration of the physical structure due to loss of organic matter.

The fact that the average wheat yield in New South Wales remains fairly constant and still largely depends on climatic factors and especially rainfall, must thus be ascribed to other factors than maintenance of soil fertility under the common system of cultivation. Firstly, the generally low yields of wheat make the nitrogen depletion of the soil a slow process. Let us imagine a soil that in a virgin condition contains 0·12% total N in the upper 6 inches, where the store of humus-N can then be estimated at 2400 lb. per acre; even if this soil for 40 years has produced an average wheat crop as high as 30 bus. every second year (which corresponds to an average consumption of about 45 lb. nitrogen), the resulting decrease in nitrogen content would be 900 lb. per acre, and the soil

[40] A small gain of nitrogen from the rain might result in seasons of actual crop failure caused by drought in the growing season and sufficient rainfall to bring down a normal quantity of nitrogen outside this period.

[41] This term might be suggested as an equivalent for the German "Raubbau".

would still contain 0·075% humus N, not even allowing for the nitrogen added by the rain or the possible utilization of nitrogen in the subsoil.[42] With an average yield of the normal 12 bushels the depletion would of course be very much slower, even on a poorer soil. Secondly, it is to be remembered that the wheat area in New South Wales has been extended greatly during the period from 1901 to 1926; this implies that large areas of new land have been taken under cultivation and thus have been subject to depletive cultivation for comparatively short times. And finally, improved methods of cultivation as well as improved varieties of wheat as regards both yield and resistance to drought and diseases, have undoubtedly done much to counterbalance the effects of decreasing humus and nitrogen content.

But it cannot be doubted that a smaller or larger loss of this nature is the general rule in soils under pure cereal-fallow cultivation—a loss which, if not remedied, must inevitably result in unproductiveness even of the rich soils in northern New South Wales. The remedy most immediately suggesting itself would be a scheme of rational cultivation of leguminous plants. Crops like field peas and soy beans are generally considered unsuitable, *inter alia*, because of their heavy demand on the soil moisture, and the ordinary fallowing system leaves only brief periods for growth of clovers and related plants (in the autumn on stubble fields, and in the spring and summer among the wheat crops, where a heavy growth is undesirable as competing with the wheat for the moisture). The only practicable way would therefore seem to be a periodical laying-down of the wheat lands to pastures sown with legumes. This laying is already, as mentioned above, practised to some extent in New South Wales, but might be improved greatly by sowing leguminous plants instead of letting them appear spontaneously (as suggested by Breakwell, 1923). The choice of the kind of legumes, the length of time that the land should be left as pasture, the extent to which it should be grazed, etc., must necessarily vary with the conditions, and must be decided by field experiments.

Where experience shows that leguminous plants will not succeed it will be necessary to resort to artificial nitrogenous fertilizers. But it must be kept in mind that inorganic nitrogenous fertilizers would merely compensate the soil for the nitrogen carried away by the crops, and their use alone in a pure cereal-fallow rotation could not be expected to lessen the danger of soil erosion due to the humus destruction which is especially rapid in warm and dry climates (Jenny, 1930), and which must always be expected during fallow. Some supply of organic matter to the soil must therefore be provided, perhaps in the form of legume-free pastures with nitrogenous fertilizers, unless it be found possible to return the straw to the soil instead of burning it.

As to the question of stubble-burning in general, this practice may probably, unless industrial uses can be found for the straw, under most conditions be continued with advantage. The waste of nitrogen which it involves is comparatively small and may be estimated at rather less than 0·4 lb. per bushel of grain produced; this could easily be made good by rational legume cultivation, or would represent only a small cost in nitrogenous fertilizers. The value of stubble-burning in checking fungal diseases is generally recognized; besides, it may be of advantage in other respects. The heating which the soil undergoes during the burning possibly acts as a partial sterilization stimulating nitrification during the

[42] Cf. the lysimeter experiments at Rothamsted (Russell and Richards, 1920) in which a soil with originally 0·146% N after leaching by rain for 47 years still produced sufficient nitrate for an annual wheat crop of 15 bus.

subsequent fallow (cf. Burgess, 1929, on the effect of grass fires on soil). If undertaken before laying the land down to pasture, stubble-burning might perhaps also be beneficial by encouraging the subsequent growth of legumes (Greene, 1935).

Field experiments on the value of these different measures in maintaining crop yields and soil fertility as well as in restoring impoverished soil must be considered a vital necessity, and should be undertaken on an extensive scale.

Summary.

The present work comprises two main sections: (1) the distribution and numbers of nitrogen-fixing bacteria in Australian wheat soils, and the nitrogen-fixing capacity in pure culture, and (2) nitrogen fixation experiments with soils under laboratory conditions, supplemented with experiments on nitrification in soils under laboratory conditions.

(1). *Azotobacter* was found in 27 out of 85 soil samples, 72 of which were taken from the wheat belt; 12% of the soils of pH 6·0 and less, and 50% of the soils of pH above 6·0, contained *Azotobacter*, mostly sporadically or in numbers less than 20 colonies per gm. of soil. Numbers of 600 to 2300 per gm. were only found in 4 soils of approximately neutral to alkaline reaction and high humus content; only one of these was a wheat soil.

Plate counts on dextrine agar gave statistically valid expressions for the density of *Azotobacter*-colonies, but Beijerinck's solution method seemed more adequate for detecting a sporadic occurrence of *Azotobacter*.

Az. chroococcum was by far the most common species. *Az. Beijerinckii* was found only occasionally and *Az. vinelandii* only once.

Anaerobic nitrogen-fixing bacteria of the butyric acid bacilli group seemed to be of almost constant occurrence in the soil.

Azotobacter was not found in the rhizosphere of wheat plants taken from soils otherwise free from *Azotobacter*. In the rhizosphere of wheat plants from an alkaline soil the numbers of *Azotobacter* were only slightly higher than in the adjacent soil, whereas the general soil microflora was far richer in the rhizosphere.

Twenty-four strains of *Azotobacter* showed a normal nitrogen-fixing capacity (9 to 18 mgm. N per gm. of glucose consumed) in pure culture. There was no evidence of a particularly economic utilization of the sugar in young cultures. Several other organisms were tested with a negative result.

(2). Nitrogen fixation experiments with soils under laboratory conditions gave the following results:

(*a*) No gain of nitrogen beyond the limits of the analytical error could be detected in 33 soils incubated for 30 days at 28–30°C. with a moderate degree of moisture, even if *Azotobacter* had multiplied vigorously during incubation. Attention is called to certain technical errors which may give rise to fictitious gains of nitrogen. A survey of the numerous statements in the literature concerning nitrogen fixation in soil incubated without addition of organic matter makes it appear questionable whether this has ever been proved with full certainty.

(*b*) Fifteen wheat soils incubated under similar conditions with addition of glucose showed mostly none or, at the best, a slight nitrogen-fixing capacity. Where nitrogen fixation took place there was always a strong multiplication of *Azotobacter* as shown by both cultural and microscopic tests; a considerable multiplication of *Azotobacter* could on the other hand take place without being accompanied by any nitrogen fixation. A control soil of alkaline reaction and high phosphate content fixed about 6 mgm. N per gm. of glucose and showed a stronger development of *Azotobacter* than any of the wheat soils. The failure

of neutral or alkaline soils to fix nitrogen on glucose addition must be ascribed to phosphate deficiency. Under anaerobic conditions, where up to 4 mgm. N per gm. of glucose could be fixed, the microscopic picture was entirely dominated by vegetative clostridia, which otherwise were hardly ever seen.

(*c*) Soils with addition of oats or wheat straw incubated under aerobic conditions for periods up to 4 months showed no gain (sometimes a loss) of nitrogen, even where the growth conditions were made optimal for *Azotobacter*, and where this organism existed for months in numbers of millions per gm. of soil. In corresponding experiments with artificial humus-free soil a fixation of 0·5–1·0 mgm. N per gm. of straw could take place. Larger gains of nitrogen were found in water-saturated media after prolonged incubation; these gains could reach 3 mgm. per gm. straw in soil after 3 months, and 6 mgm. per gm. straw in and after 8 months. This seems to be due to a more copious formation of organic acids, etc., as metabolic by-products in the anaerobic decomposition of the straw in the water-saturated medium, which by-products are utilized by *Azotobacter* on the surface of the medium; clostridia were little in evidence under these conditions. In fully aerated soil only the water-soluble constituents of the straw become available to *Azotobacter*.

Experiments with root-material of wheat gave results not essentially different from those found with straw. A high degree of soil moisture seems generally necessary for an effective utilization of cellulosic materials for nitrogen fixation.

Where nitrogen fixation took place it was, as in the experiments with glucose, always accompanied by a multiplication of *Azotobacter*, which reached numbers incomparably higher (ten thousands to millions per gm. of soil) than ever found under field conditions. Generally the numbers of this organism, as revealed by both microscopic and plate counts, were so high as to suggest that the process of nitrogen fixation consisted in a simple synthesis of *Azotobacter*-cells; only a few experiments with artificial soil gave some suggestion of small gains of nitrogen through other processes.

The most efficient nitrogen fixation observed in soil was 14–15 mgm. N per gm. of glucose and 5–8 mgm. per gm. of acetic or lactic acid, which is very similar to the yields in vigorously fixing pure cultures of *Azotobacter*. There is thus no evidence that *Azotobacter* will necessarily utilize its nutrients more economically in the soil than in pure cultures.

(*d*) Wheat soils exposed to daylight for periods of 3 to 4 months showed no gains due to the development of algae. In experiments with another soil and with sand medium, small quantities of nitrogen were fixed apparently by blue-green algae. Even the highest gains of nitrogen in the cultures would correspond to only small quantities in the field, where the conditions are rarely favourable for nitrogen fixation by algae; this process cannot therefore be credited with any significance in the wheat soils.—It cannot be regarded as proved that nitrogen is fixed in the soil by photochemical processes, as maintained by Indian investigators.

(*e*) Fifty-five soils of different character, including 46 wheat soils, showed after incubation for 30 days at 28–30°C. a production of nitrate and ammonia roughly proportional to the nitrogen content of the soil. In the wheat soils the correlation coefficient between produced (NO_3 + NH_4)—N and total soil nitrogen amounted to +0·625. From 0·1 to 5·2, in most cases 1·5 to 3·0% of the initial organic ("humus") nitrogen was converted into mineral N, chiefly nitrate; there was little or no accumulation of ammonia.

As a general conclusion there seems to be no foundation for the frequently expressed opinion that soils from arid climates have an extraordinary nitrogen-fixing power and may, through the utilization of crop residues by free-living nitrogen-fixing organisms, be permanently under cereal cultivation without depletion of nitrogen. In Australian wheat soils we can normally expect no gain at all and only under exceptionally favourable circumstances a fixation corresponding to one-third of the nitrogen requirements of the crops on wheat land worked on the usual wheat-fallow rotation. The importance of non-symbiotic nitrogen fixation in nature is probably largely confined to uncultivated soils where no crops are carried away and the vegetable debris is allowed to decompose *in situ*.

The practice of growing wheat alternating with fallow and without use of nitrogenous fertilizers must be regarded as a gradual consumption of the nitrogen reserves of the soil, from which some nitrate is produced during fallowing. This consumption is only incompletely compensated by non-symbiotic nitrogen fixation and the effect of the rain, and if continued it must in time lead to permanent loss of fertility. The natural remedy must be looked for in the introduction of leguminous crops in the rotations, or a judicious application of nitrogenous fertilizers where this is not practicable. Investigations into these problems as well as the possible importance of wild legumes on the wheat fields are urgently required.

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EXPLANATION OF PLATE I.

Figs. 1-2.—Plate counts of *Azotobacter* on dextrine agar. Inc. 6 d. 28-30°C. 1, normal soil (No. 44, Table 3, 20/10/1937), dil. 1 : 50; 2, soil with addition of wheat straw plus 0·5% Ca-acetate (Table 18), dil. 1 : 1,000,000. (Arrows indicate colonies resembling the "bacille gommeux" of Winogradsky, 1926.)

Fig. 3.—*Azotobacter*-like organisms, small type, from drop-film; soil No. 8 + glucose, inc. 4 d. 28-30°C. after 2nd addition of glucose.

Fig. 4.—Ditto, big type; from soil No. 10 + glucose and sodium phosphate, inc. 4 d. 28-30°C. after 1st addition.

Fig. 5.—Ditto, very small type; soil No. 32 + filter paper and Ca-lactate, inc. 7 d. 28-30°C.

Fig. 6.—*Azotobacter* from surface colony, soil No. 31 + 33 + wheat straw and calcium carbonate, high moisture content, inc. 3 d. 28-30°C.

Fig. 7.—Clostridia; soil No. 21 + glucose, anaerobic, inc. 7 d. 28-30°C.

Fig. 8.—Blue-green algae; impression preparation from surface of soil No. 8, inc. 90 d. in daylight.

Fig. 9.—Nitrogen-fixing blue-green algae (*Anabaena* sp.?), from culture in mineral solution, inc. 8 weeks in daylight.

Staining: Figs. 3-6, rose bengale; Fig. 7, Gram plus rose bengale; Fig. 8, erythrosine; Fig. 9, unstained.

Magnification: Figs. 3-8, × 625; Fig. 9, × 290.